



## Microalgae biorefinery symbiosis: screening, production, and process analytical technology

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# Microalgae biorefinery symbiosis: screening, production, and process analytical technology

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PhD Thesis  
May 2017

DTU Environment  
Department of Environmental Engineering  
Technical University of Denmark

Microalgae biorefinery symbiosis: screening, production, and process  
analytical technology

**Michael Podevin**

PhD Thesis, June 2017

The synopsis part of this thesis is available as a pdf-file for download from  
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# Preface

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductory review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-III**.

- I Podevin, M.**, De Francisci, D., Holdt, S.L., Angelidaki, I., 2015. Effect of nitrogen source and acclimatization on specific growth rates of microalgae determined by a high-throughput in vivo microplate autofluorescence method. *J. Appl. Phycol.* 27, 1415–1423. doi:10.1007/s10811-014-0468-2
- II Podevin, M.**, Fotidis, I.A., De Francisci, D., Møller, P., Angelidaki, I., 2017. Detailing the start-up and microalgal growth performance of a full-scale photobioreactor operated with bioindustrial wastewater. *Algal Res.* 25, 101–108. doi:10.1016/j.algal.2017.04.030
- III Podevin, M.**, Fotidis, I.A., Angelidaki, I., 2017. Microalgal process-monitoring based on high-selectivity spectroscopy tools: status and future perspectives. (Submitted to *Critical Reviews in Biotechnology*)

In this online version of the thesis, paper **I-III** are not included but can be obtained from electronic article databases e.g. via [www.orbit.dtu.dk](http://www.orbit.dtu.dk) or on request from DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, [info@env.dtu.dk](mailto:info@env.dtu.dk).

This PhD study also contributed to international conferences with the following proceeding papers:

- **Podevin, M.**, Borch, M.M., De Francisci, D., Holdt, S.L., Angelidaki, I., Møller, P., 2012. Screening and Optimization of Case Specific Sustainable Mixotrophic Microalgal Medium. Young Algaeneer Symposium, Wageningen, Netherlands, 14/06/2012 - 16/06/2012,
- De Francisci, D., Holdt, S.L., Van Wagenen, J., **Podevin, M.**, Smets, B.F., Plósz, B., Møller, P., Angelidaki, I., 2013. Development of an algal wastewater treatment concept, based on the selection of microalgal strains with optimal bioextraction characteristics. International Conference on Algal Biorefinery, Kharagpur, India, 10/01/2013 - 12/01/2013,
- Van Wagenen, J., De Francisci, D., Valverde Perez, B., Holdt, S.L., **Podevin, M.**, Smets, B.F., Plósz, B.G., Møller, P., Angelidaki, I., 2013. Microalgae Biorefinery - Industrial Symbiosis. Copenhagen Bioscience Conference: Cell Factories and Biosustainability, Denmark, 03/11/2013 - 06/11/2013,

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To Karen: I am very happy that the only thing you had to do with this thesis was distracting me from it in the best ways possible.



# Summary

Microalgae treatment of municipal wastewater (WW) has been the focal point of microalgal biotechnology research for several decades. Microalgal WW treatment has made a resurgence with the idea of using biomass from microalgal WW treatment, as a source of lipids for conversion into biodiesel. In recent years, microalgal research continued with the prospect of a microalgae biorefinery, where microalgal byproducts and coproducts are extracted to valorize the entire microalgal production. The concept of an “industrial symbiosis” has also emerged in the past several decades, in which networks of industries cooperate to use waste sources from neighboring industries, in industrial parks, to create added value. The intersection of the microalgae biorefinery and industrial symbiosis, in a microalgae biorefinery symbiosis (MBS), may be the next generation scheme to valorize the microalgal production and promote industrial and global sustainability. Moreover, technological advances in screening, outdoor photobioreactor (PBR) design, macromolecular monitoring and process automation must all be addressed to execute the complex bioprocesses needed to valorize an MBS successfully.

In order to properly identify viable MBS partnerships with industry, microalgal species capable of producing an array of valuable products must first be screened on these potential bioindustrial WW streams for their growth potential. During screening, microalgae may have a preference or aversion for a given bioindustrial WW media, based on the types and ratios of nitrogen (ammonium, nitrate, or urea) in the WW. Furthermore, identifying algae capable of withstanding fluctuations between these nitrogen forms in dynamic WWs, is an important criterion for productivity. However, when screening microalgae on WWs containing different nitrogen sources and concentrations, assimilation of different nitrogen sources can result in starkly different physiochemical changes, specifically pH changes. By growing batch cultivations of microalgae in 24-well microplates, a microplate reader can be used to measure relative fluorescence of chlorophyll *in vivo*, during balanced growth, before these pH changes occur. Additionally, along with being spatially high-throughput in a 24-well microplate, the early and low detection of growth rates is also more temporally high-throughput than any other microalgae screening method.

From microplate scales to large-scales—six orders of magnitude larger—the industrially important screened microalgae *Chlorella sorokiniana* was grown



on bioindustrial WW, inside a novel full-scale, solar tracking, 4000 L, airlift PBR. Despite cold temperatures and low irradiance, the mesophilic microalgae reached a growth rate of  $0.48 \text{ day}^{-1}$ , in the four-day period immediately following inoculation of bioindustrial WW containing ammonium, as a primary nitrogen source. After that, after ammonium was depleted and the media was augmented with nitrate, a long lag phase persisted, before undergoing the predominant production phase with a specific growth rate (SGR) of  $0.15 \text{ day}^{-1}$  over an 18-day period. Moreover, it was demonstrated that microalgae could grow in adverse environmental conditions at large-scales, without any significantly evident deleterious effects of the bioindustrial WW on microalgae growth.

The success of the *in vivo* fluorescence microplate assay and the complexity of these outdoor reactions demonstrate the value of pursuing real-time data of microalgae *in vivo* at large-scales. Also, the complex and dynamic nature of large-scale outdoor microalgal reactions, when grown on dynamic WW media, encourages the need for on-line, real-time monitoring to improve automation models of PBRs. Recent advances in hardware and software can significantly improve microalgal bioprocess models and automation, by machine learning manipulation large, time-resolute data sets, so-called “big data,” which can be acquired through high-selectivity vibrational spectroscopy, such as mid-infrared (MIR), near-infrared (NIR), or Raman vibrational spectroscopies. With microalgae entering a new paradigm of food, feed, pharmaceuticals and functional products, on top of biofuels in a biorefinery, there will be a growing need to maintain product quality, regulate, and mitigate contamination, especially in a symbiosis with WW. Furthermore, vibrational spectroscopies can be used to monitor several microalgal components simultaneously, which can be used to aid fractionation of microalgal compounds in a biorefinery, while improving model building for automation and control of product quality and contamination, where quality can be built into the system.

The results and research summarized in this thesis demonstrate that the modernization of microalgal research is becoming increasingly necessary and beneficial to microalgae production in an MBS. The focus of this thesis is to bring together lab-scale demonstrations, scaled up knowledge, and a critical outlook of modern technologies capable of making the MBS a reality at the production step of a biorefinery.

# Dansk sammenfatning

Kommunal spildevandsrensning med mikroalger har været et omdrejningspunkt indenfor bioteknologisk forskning i mikroalger i flere årtier. Ved ideen om at anvende mikroalgebiomassen fra spildevandsrensning som en kilde til lipider, der kan omdannes til biodiesel, er spildevandsrensning med mikroalger blevet genoplivet. I de seneste år er forskning i mikroalger fortsat, med udsigt til et mikroalgebioraffinaderi, hvor ekstraherede bi- og coprodukter fra mikroalgeproduktionen skaber en større omsætning end selve mikroalgeproduktionen. Konceptet om en "industriel symbiose" er ligeledes opstået i de seneste årtier, hvor netværk af industrier samarbejder om at bruge affaldskilder fra nærliggende industrier i industri-parker, for at skabe merværdi. Skæringspunktet mellem mikroalgebioraffinaderier og industriel symbiose i en mikroalgebioraffinaderisymbiose (MBS) kan være den næste generation af forretningsmodel til at skabe merværdi i mikroalgeproduktionen og fremme dens industrielle og globale bæredygtighed. Desuden skal teknologiske fremskridt indenfor screening, design af udendørs fotobioreaktor (PBR), makromolekylær overvågning og procesautomation alle håndteres, for at kunne udføre de komplekse bioprocesser, der er nødvendige for at værdiskabe en MBS.

For at kunne identificere et levedygtigt MBS-partnerskab, skal mikroalgarter, der er i stand til at producere en vifte af værdifulde produkter, først screenes på disse potentielle bioindustrielle spildevande. Under screening kan mikroalger have en præference eller modvilje for en given bioindustriel spildevand, baseret på type og fordeling af nitrogen (ammonium, nitrat eller urea) i spildevandet. Desuden er identifikation af alger, der er i stand til at modstå svingninger mellem disse nitrogenformer i dynamiske spildevande, et vigtigt produktionskriterie. Ved screening af mikroalger i spildevande, der indeholder forskellige nitrogenkilder og koncentrationer, kan assimileringen af forskellige nitrogenkilder imidlertid resultere i markant forskellige fysiokemiske ændringer, specielt pH-ændringer. Ved at batchdyrke mikroalger i 24-brønds mikropalader kan en mikropaladelæser anvendes til at måle relativ fluorescens af klorofyl *in vivo* under afbalanceret vækst, før disse pH-ændringer opstår. Udover at give en rumlig produktionsmængde i en 24-brønds mikropaladeer den tidlige og lave detektering af vækstrater også med til at give en højere tidsmæssigt produktionsmængde end nogen anden mikroalgscreeningsmetode.

Fra mikroplade skala til større skala - seks størrelsesordener større – er den industrielt vigtige mikroalgen *Chlorella sorokiniana* blevet dyrket i industriel spildevand, inde i en ny, solstyret, 4000 L luftløftet PBR. På trods af kolde temperaturer og lav bestrålingsstyrke nåede den mesofile mikroalge op på en vækst på  $0,48 \text{ dag}^{-1}$  i en fire-dages periode umiddelbart efter inokulering med bioindustriel spildevand, der indeholder ammonium som den primære nitrogenkilde. Derefter, efter at ammonium er blevet opbrugt og mediet forstærket med nitrat, er der en langt inaktiv periode, inden den gennemgår den største vækstfase med en specifik vækstrate på  $0,15 \text{ dag}^{-1}$  over en 18-dags periode. Endvidere viste dette, at mikroalger kan vokse i ugunstige miljømæssige forhold på stor skala, uden nogen væsentligt tydeligt skadelige virkninger af bioindustriel spildevand på mikroalgevækst.

Succesen af *in vivo* fluorescens mikropladetest og kompleksiteten af disse udendørsreaktioner viser værdien af, at forfølge real-time data af mikroalge *in vivo* på stor skala. Den komplekse og dynamiske natur af store udendørs mikroalgereaktioner, når de dyrkes på dynamiske spildevandsmedier, opfordrer til behovet for online overvågning i realtime for at forbedre automatiseringsmodeller af PBR'er. De seneste fremskridt inden for hardware og software kan imidlertid forbedre modeller for mikroalgebioprocesser og automation betydeligt ved maskinindlæringsmanipulation af store tidsbesparende datasæt, såkaldte "big data", som kan erhverves gennem høj-selektivitetens vibration spektroskopi. Med mikroalger, der indgår i et nyt paradigme af mad, foderstoffer, lægemidler, funktionelle produktioner samt biobrændstoffer i et bioraffinaderi, vil der være et voksende behov for at opretholde produktkvalitet, regulere og mindske forurening, specielt i en symbiose med spildevand. Endvidere kan vibrations spektroskopi bruges til at overvåge flere mikroalgekomponenter samtidigt, som kan bruges til at hjælpe fraktionering af mikroalgeforbindelser i et bioraffinaderi, samtidig med at man forbedrer modelbygningen til automatisering og kontrol af produktkvalitet og forurening, hvor kvalitet kan indbygges i systemet.

Resultaterne og forskningen opsummeret i denne afhandling viser, at moderniseringen af mikroalgeforskning bliver mere og mere nødvendig og gavnlig for mikroalgeproduktioner i en MBS. Fokuset for denne afhandling er at samle laboratorieskala demonstrationer, opskaleret viden og et kritisk syn på moderne teknologier, der kan gøre MBS til en realitet på produktionstrinnet af et bioraffinaderi.

# Table of contents

<b>Preface.....</b>	<b>iii</b>
<b>Acknowledgements .....</b>	<b>v</b>
<b>Summary .....</b>	<b>vii</b>
<b>Dansk sammenfatning .....</b>	<b>ix</b>
<b>Table of contents .....</b>	<b>xi</b>
<b>Abbreviations.....</b>	<b>xiii</b>
<b>1 Introduction.....</b>	<b>1</b>
1.1 Industrial and global sustainability and renewable economies .....	1
1.2 Modernizing microalgal research and modeling.....	2
1.3 Objectives and thesis structure.....	4
<b>2 Microalgae biorefinery symbiosis (MBS) .....</b>	<b>9</b>
2.1 Microalgal bioproducts in a biorefinery .....	9
2.2 Microalgae in an industrial symbiosis .....	11
<b>3 Microalgae process analytical technology (PAT) and automation ....</b>	<b>17</b>
<b>4 Screening of microalgae.....</b>	<b>21</b>
<b>5 Full-scale PBR microalgae production on bioindustrial wastewater .</b>	<b>29</b>
5.1 Photobioreactor design .....	29
5.2 Bioindustrial wastewater composition and pretreatment .....	30
5.3 Microalgae growth on bioindustrial wastewater .....	33
5.4 Harvesting with microfiltration.....	35
<b>6 Towards modeling complex large-scale photobioreactors .....</b>	<b>37</b>
<b>7 Conclusions.....</b>	<b>49</b>
<b>8 References.....</b>	<b>51</b>
<b>9 Papers .....</b>	<b>65</b>



# Abbreviations

CQA	Critical Quality Attributes
CPP	Critical Process Parameters
HVP	High-Value Product(s)
IC	Internal circulation
PBR	Photobioreactor
MIR	Mid-Infrared
NIR	Near-Infrared
SGR	Specific Growth Rate
WW	Wastewater



# 1 Introduction

## 1.1 Industrial and global sustainability and renewable economies

For decades, microalgal feedstocks have been looked at for their high lipid yields for biofuels and their ability to assimilate nutrients and oxidize and assimilate organic matter in wastewater (WW). However, the valorization of the production and harvesting of microalgae for biofuels, even on free nutrients from WWs, can still not compete with the price of crude oil (NAABB, 2014; Wijffels and Barbosa, 2010). Meanwhile, microalgal biotechnology has gained more attention because of the ability of microalgae to synthesize several valuable bioproducts, that may give microalgae biotechnology a competitive advantage as an environmentally friendly and sustainable alternative to a number of industrial products. While the production of microalgae lipids for biofuels cannot yet be economically justified, a biorefinery of several products from microalgae can offset the costs of constructing, maintaining, harvesting, and refining a microalgae bioreaction (Subhadra and Grinson-George, 2011; Subhadra, 2010; Wijffels et al., 2010). In a biorefinery concept, lipids, proteins, and carbohydrates can be directed to various markets (Vanthoor-Koopmans et al., 2013) and potential high-value product (HVP) markets for pigments, fatty acids, sterols, and other products can also significantly offset capital costs (Borowitzka, 2013).

In principle, biorefining microalgae begins with the fractionation of lipids, carbohydrates, protein, and residual biomass, followed by further refining and selling of components for added value (Foley et al., 2011; Pragya et al., 2013; Subhadra and Grinson-George, 2011; Vanthoor-Koopmans et al., 2013). Additionally, following extractions of HVP, residual components can be directed to several bioenergy technologies, not limited to biodiesel, such as biogas and bioethanol (Foley et al., 2011; Koller et al., 2014; Trivedi et al., 2015). Furthermore, the principles of microalgae biorefinery can also be approached to address several growing societal concerns depending on the desired outcome. These concerns include sustainability, carbon mitigation (Farrelly et al., 2013), water treatment and re-use (Craggs et al., 2011), food security (Brune et al., n.d.; Cordell et al., 2009; Walsh et al., 2015), phosphorus removal recovery (Cordell et al., 2009; Shilton et al., 2012), heavy metal pollutant removal and recovery (Wilde and Benemann, 1993), bioenergy (Bohutskyi and Bouwer, 2013; Collet et al., 2011; Markou et al., 2012; Montingelli et al., 2015), animal



feed (Subhadra and Grinson-George, 2011), off-setting the loss of genetic diversity of terrestrial plants (Campbell and Doswald, 2009; Subhadra and Grinson-George, 2011), nutrient rich coproduct for feeding people in underdeveloped areas (Subhadra and Grinson-George, 2011; Subhadra, 2010), and even space exploration life support systems (Smirnova and Mariia, 2016; Wagner et al., 2015). Furthermore, when free nutrient resources are available in waste streams from industry, which otherwise require WW treatment, some industries may find an economic advantage in growing microalgae to valorize their wastes in what is referred to as an industrial symbiosis. The intersection of the microalgae production, biorefinery, and a symbiosis where algae are grown on bioindustrial WW media will be referred to as a microalgae biorefinery symbiosis (MBS). Moreover, with its many avenues towards sustainability, microalgae have been and may continue to be the most important organisms to human life.

## 1.2 Modernizing microalgal research and modeling

This thesis work addresses various challenges and pivotal steps aimed at improving large-scale, outdoor microalgae productions in a biorefinery setting using WW as growth media. The complexity of large-scale, outdoor microalgae biorefineries using WW, shift academic concerns towards novel technological approaches for screening microalgae and microalgal production process control to maintain HVP quality.

Currently, common screening techniques are laborious and do not consider the effects that various types, concentrations, and proportions of nitrogen sources may have on microalgal growth. With over 40,000 species of algae (Guiry, 2012) with unique phenotypic responses to environments, high-throughput microalgal screening is the natural solution to finding a candidate microalgal species for production. Recently, microplate screenings have been used to identify microalgae capable of quickly adapting to new conditions, such as varying nitrogen sources (Podevin et al., 2015) and industrial WW (Van Wagenen et al., 2014). Microplates can also be used to estimate key parameters in productivity models to be used in scaled up experiments. Van Wagenen et al. (2014b) demonstrated that the specific growth rate (SGR) vs. light intensity dependency ( $\mu$ -I curve), a key parameter in productivity models, could be determined in microplates. After that, scaled up, bench scale photobioreactors (PBRs) can be used to build mathematical models and simulations to infer other model parameters to improve productivity at large-scales.

Currently, most microalgae models address the optimization of productivity and the biosynthesis of primarily lipids and pigments; however, in a large-scale MBS, different models for biosynthesis and product yields of HVPs are necessary to maintain the quality of these HVPs, especially if grown on dynamic WWs in an industrial symbiosis. On one hand, first principles stoichiometric metabolic flux models can be used to estimate a number of metabolites by prescribing likely metabolic pathways, from knowledge about carbon allocation to analysis and the microalgal genome during balanced growth (Cogne et al., 2003; Kliphuis, 2012; Shastri and Morgan, 2005; Yang, 2000). However, during balanced growth, metabolite storage and various kinetic parameters are not accounted for, except for, in some cases, transient light conditions (Bernard et al., 2015). Furthermore, outdoor PBR light and temperature fluctuations and dynamic media composition make balanced growth, and by proxy metabolic modeling, extremely difficult. On the other hand, simpler, semiempirical, so-called “compartmental models” (e.g. Droop models), are capable of modeling empirical kinetic features and biomolecular quotas of microalgae to provide a physiological status. Overall, since transient light conditions and media (e.g. WW) in outdoor PBRs can affect microalgae at small time scales, both model types are still very limited for the complex reactions taking place to create a profitable MBS.

In large-scale, outdoor PBRs, models based on as little input as pH, temperature, solar insolation, cell density, may not be sufficient for understanding the allocation of carbon inside microalgal cells, especially where many of these models are built around steady state assumptions, balanced growth, and ideal mixing. These assumptions often preclude the effects or combined effects of diel, diurnal, transient and gradient light and temperature variations, non-ideal mixing, not to mention the composition and other physical properties (e.g. viscosity) of WW (Bernard et al., 2015; Celikovskiy et al., 2010; Grogan et al., 2010; Havlik et al., 2013). For example, the light reactions of microalgae take place on the order of milliseconds and in some cases light patterns from hydrodynamics can take place in microseconds (Bernard et al., 2015; Posten, 2009). Similarly, photodamage can occur in a matter of minutes compared to days in which growth and photoacclimation processes occur (Bernard et al., 2015). Moreover, fast time-scale models capable of characterizing the effects of light and hydrodynamic light regimes (e.g. “flashing light effect”) are likely the best methods to model and optimize productions at industrial scales. Fur-

thermore, such a model would have to be coupled with nutrient models in fluctuating WW media. Ultimately, several models would be needed at various time-scales to predict productivity of biomass and HVP in an MBS.

It follows that microalgal productions are in need of modern technological advances to create models with the robustness of compartmental models and with the specificity of metabolic flux models in an MBS. Developments in hardware and software, and their intersection in bioindustry may be directed to augment existing models or creating entirely new modeling approaches:

- **Hardware** developments of on-line sensors capable of detecting several intracellular and intercellular compounds in real-time. Prospectively, these technologies can also differentiate microalgal species, strains, and phenotypic variations on-line or in-line in outdoor PBRs.
- **Software** developments have paved the way for new, multivariate models to improve process automation. By taking rich real-time information—big data—about carbon allocation and HVP content inside microalgal cells and relating it to known, real-time PBR parameters, very robust models can be created. These models are capable of being adaptive (i.e. artificial neural networks) and can be used to develop a “fingerprint” for the highly dynamic and complex bioreactions needed to implement an outdoor MBS.

### 1.3 Objectives and thesis structure

The overall objective of the work outlined in this thesis is to further the understanding of the practicality and plausibility of a sustainable and financially realistic microalgae biorefinery symbiosis (MBS). This thesis entails a summary of research dedicated to the critical steps towards the realization of an MBS from optimization of microtiter lab screening to full-scale production and the prospects of full-scale modeling and automation.

The first objective of the work contained in this thesis was to understand if *in vivo* autofluorescence of microalgae could be used to observe SGRs of microalgae before large physiochemical changes occurred in the media, specifically pH. Furthermore, it will be demonstrated that this early detection method may be better than scaled up experiments, where large pH changes are expected to change, and may even mirror scaled-up batch experiments where pH is kept constant. Furthermore, the work addressed in this objective has been

done in the interest of screening microalgae on WWs, which can contain several nitrogen sources (paper I).

The second objective was to use the newly establish screening method to determine if accurate nitrogen source preferences could be established for four industrial important unicellular microalgae. This objective will also reiterate that highly sensitive autofluorescence detection can be used to screen the specific growth rate (SGR) of a given microalgae before physiochemical changes due to various nitrogen assimilation pathways occur in the media. In this objective, the adaptability of the four microalgae to new environments will also be considered for screening of strains with rapid acclimatization potential to be used in scaled up experiments and WW screening (paper I).

The third objective was to use a high specific SGR microalgae determined during screening and scale its production directly up to a full-scale reactor module. Microplate screening indicated that microalgae with minimal adaptation periods and high SGRs in any nitrogen source have the best potential to be grown on treated bioindustrial WW that contained several different nitrogen sources. WW media purification through ultrafiltration and microalgae harvesting through microfiltration were also considered to meet the simulate major components of an MBS (paper II).

The fourth objective of this work was to research and identify novel tools capable of real-time monitoring of highly inhomogeneous and dynamic microalgae productions for quality control of high-value products in an MBS. High-selectivity, on-line/in-line, real-time vibrational spectroscopies were reviewed to highlight the potential benefits that these spectroscopies might have on bioreaction automation in the advent of modern technological improvements in hardware, software, and machine learning (paper III).

Overall, this thesis will overview the nuances of spectroscopy at lab-scale (paper I), full-scale (paper II), and at a theoretical and academic level (paper III), as they pertain to all levels of this thesis. Illustrated in Figure 1.

The interplay of the main objectives of this thesis work and their relevance to the realization of an MBS amid regulatory, quality, and practical concerns will be addressed in the following chapters:

Chapter 2, highlights the promise of microalgae as a lucrative feedstock for an MBS because of its diversity of species and products of high-value.

Chapter 3 contains a discussion of the terminology and the need for process and quality control as well as automation for a highly dynamic MBS to create products consistent with market standards.

Chapter 4 contains an overview the development of a microplate screening assay to test various microalgae grown on different nitrogen sources to simulate screenings of microalgae grown on WW media in an MBS. This chapter will also address the screening of microalgae capable of rapid adaptation to new nitrogen sources, which may fluctuate in WW media. Furthermore, the physiochemical boundaries of microplate measurements, as well as the benefit of low detection chlorophyll autofluorescence in microalgae will also be addressed.

Chapter 5 contains an overview of growing robust screened microalgae at large-scales, outdoors, in a novel photobioreactor (PBR). This chapter also contains upstream treatment of a bioindustrial WW and downstream harvesting of the microalgae to simulate, in part, a full-scale MBS.

Chapter 6 contains a discussion and assessment of mid-infrared (MIR), near-infrared (NIR), and Raman vibrational spectroscopies: high-selectivity, on-line/in-line monitoring tools that are capable of monitoring multiple analytes including lipids, proteins, carbohydrates, as well as high-value products such as pigments. The discussion will be focused on using on-line real-time monitoring and automation to optimize microalgae bioreactions for quality control as well as creating a profile of the microalgae for proper fractionation and extraction of the array of chemicals in MBS.

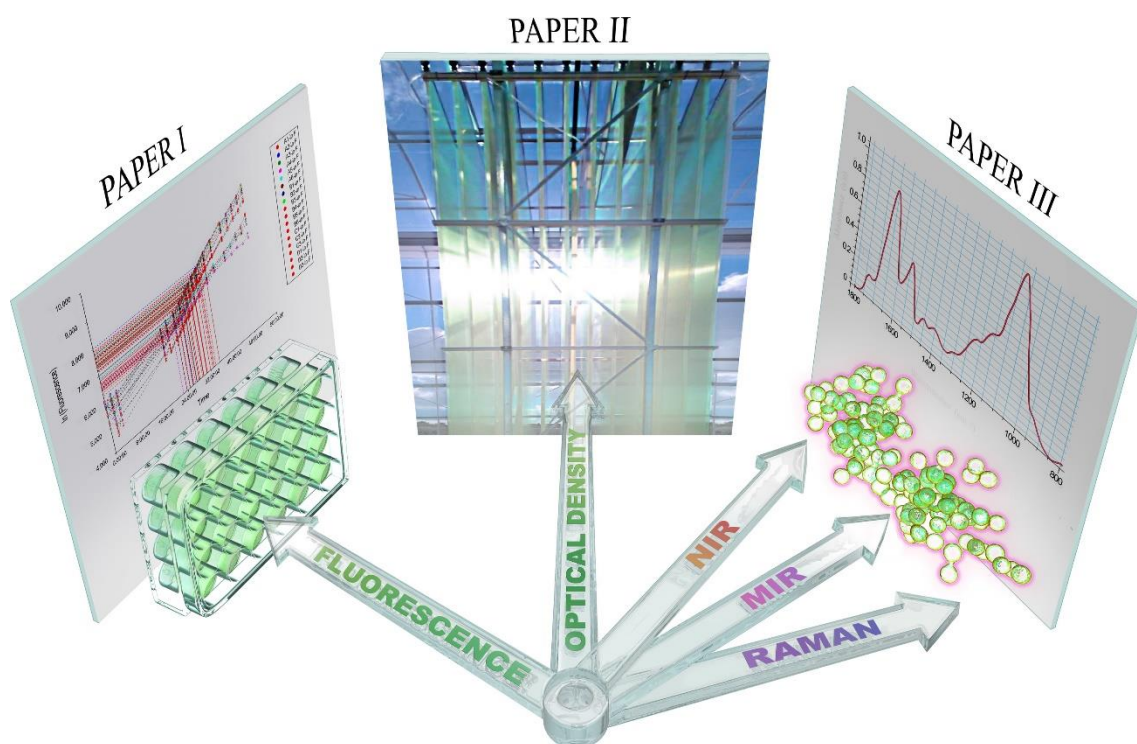


Figure 1. Paper I addresses a novel microplate fluorescence spectroscopy method for screening microalgae. Paper II addresses the viability of optical density spectroscopy during operation of a full-scale PBR. Paper III contains a critical review of the theory and recent applications of NIR, MIR, and Raman vibrational spectroscopies for determining various microalgal compounds.



## 2 Microalgae biorefinery symbiosis (MBS)

### 2.1 Microalgal bioproducts in a biorefinery

There are many thousands of species of microalgae and cyanobacteria, which contain numerous valuable bioproducts, some of which can be used to valorize microalgae productions. In all microalgae species, there are four broad chemical compounds: lipids, proteins, carbohydrates, and nucleic acids. In most microalgae, lipids and proteins make up the majority of biomass, followed by carbohydrates and nucleic acids (Becker, 1994; E. W. Becker, 2013; W. E. Becker, 2013). There are a variety of high-value chemicals that can come from microalgae and some remain unexplored due to the diverse phylogeny of microalgae with over 40,000 species of *identified* algae (Borowitzka, 2013; Guiry, 2012).

Currently, the gamut of possible microalgal metabolites and whole microalgal products spans food, feed, fuel, pharmaceuticals, nutraceuticals, and many other functional products (E. W. Becker, 2013; W. E. Becker, 2013; Borowitzka, 2013; Enzing et al., 2014; Vigani et al., 2015). However, the global market demand has also played a crucial role in the prevalence of only a few microalgae species in productions for human consumption. As of 2014, there are several microalgal products that are either commercialized or in advanced development according to an extensive survey performed by Enzing et al. (2014). According to their research, long chain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) omega-3 polyunsaturated fatty acids (PUFAs),  $\beta$ -carotene, phycocyanin, astaxanthin, and whole form microalgae such as *Spirulina*, *Chlorella*, *Nannochloropsis*, and *Isochrysis* are already in a commercialization pipe-line. Other essential PUFAs of commercial interest for food and feed not currently in a pipeline include: various linoleic acids, linolenic, and arachidonic acid; however, many of these PUFAs cannot currently compete with fish oil derived PUFA prices (E. W. Becker, 2013). On the other hand, there are numerable other microalgae used as feed in aquaculture of mollusks, shrimp, abalone, rotifers, copepods, and zooplankton, in which 40% of aquaculture depends on commercial microalgal feeds, where some (e.g. salmon) are 100% dependent on commercial feeds (E. W. Becker, 2013).

Aprart from high value lipids, microalgae are also rich in vitamins A, B1, B2, B6, C, E, K, niacin, biotin, folic acid, pantothenic acid, and other valuable pigments such as lutein and zeaxanthin can also be directed to the marketplace;



however, many of these vitamins are unstable and may be compromised during post-harvesting extraction (W. E. Becker, 2013). Despite the commercial realization of single products or whole microalgae, there is little knowledge about the commercial realization of a biorefinery diverting byproducts and coproducts during the extraction and fractionation of these high-value chemicals. Generally speaking, in a biorefinery concept, it is understood that the value of the sum of the parts of microalgae may be more valuable than the whole microalgae. The valorization of microalgae productions through a biorefinery can also be promoted by other economic drivers, such as WW treatment, which can be realized in an industrial symbiosis and ultimately in a microalgae biorefinery symbiosis (MBS). The breakdown of broadly categorized economic drivers, which include products for food and feed, biofuels, as well as offsetting wastewater (WW) treatment (oxygen aeration of organic matter and N removal) can be seen in Figure 2.

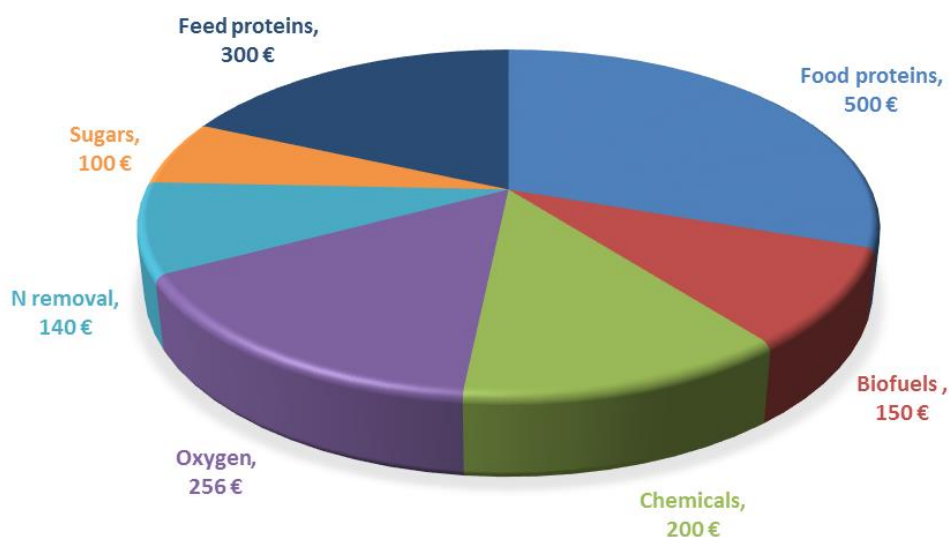


Figure 2. Microalgal biomass biorefinery price (EUR) per 1000 kg. Figure adapted from Wijffels et al. (2010).

In the valorization of an MBS, biogenic toxins should also be considered during production. Nucleic acids themselves, which constitute approximately 4-6% of microalgal dry weight, have the potential to increase plasma uric acid concentration in humans through the ingestion of purines leading to gout and kidney stones. Similarly, it has even been suggested that the threshold for beneficial and adverse effects on humans of the commercially sought after bioproduct  $\beta$ -carotene is low (Omenn et al., 1996). Also, cyanobacteria produc-

tions have gained some notoriety for producing toxic substances from contaminating cyanobacterial strains, which can produce hepatotoxins (Yang et al., 2011), fatal anatoxins (neurotoxins) (Rellán et al., 2009). The regulatory elements of microalgae production have been reviewed (Carmichael, 1994; Enzing et al., 2014; Vigani et al., 2015; Yang et al., 2011); however, these very important regulatory factors are often viewed as a footnote to the broader engineering challenge of making a viable and economical microalgae production. Moreover, in a biorefinery, microalgae PBRs may need to be monitored in real-time to maintain consistent product quality to mitigate these concerns and safely implement and valorize the MBS through regulatory competence.

## 2.2 Microalgae in an industrial symbiosis

Currently, the philosophy of an “industrial symbiosis” has been put to the test at the Kalundborg Symbiosis (Kalundborg, Denmark) where various waste products from industry are directed to other industry in the vicinity; cooperating to get added value out of a multitude of waste streams. In the symbiosis, water, materials, energy, industrial byproducts can be directed to various nearby industry to give the involved parties a competitive advantage industrially, while financially incentivizing sustainability. A schematic of the Kalundborg industrial symbiosis can be seen in Figure 3.

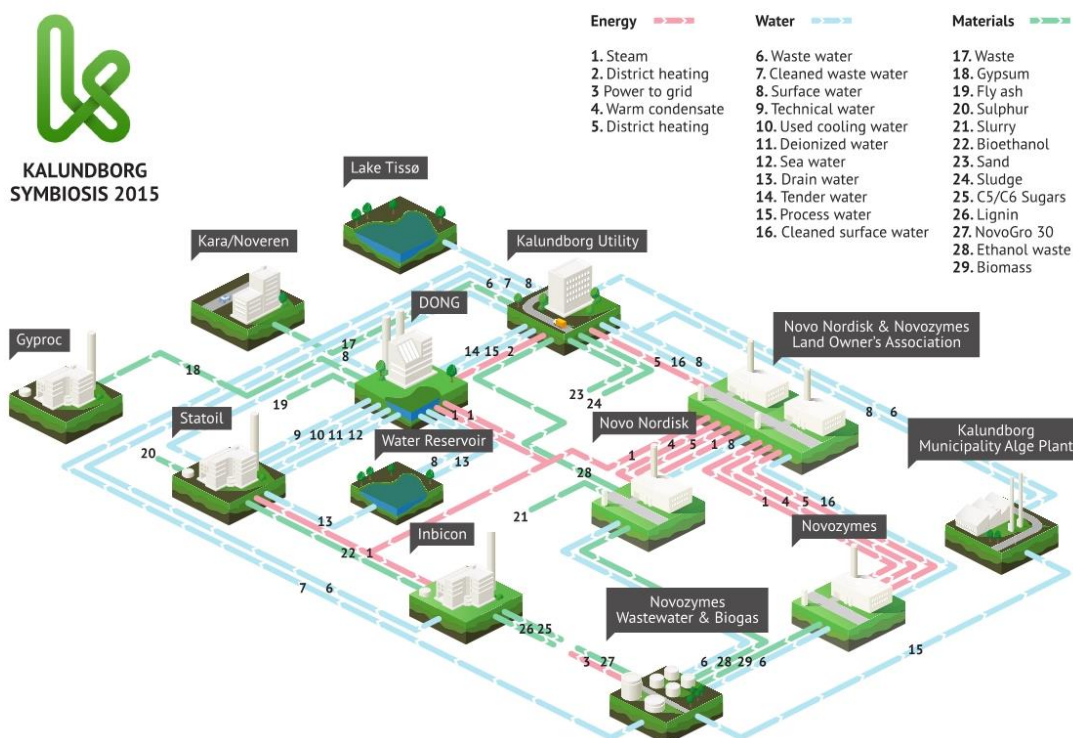


Figure 3. Kalundborg industrial symbiosis (Kalundborg, Denmark) with energy, water, and material streams exchanged between several industries. Figure provided by Per Møller (Kalundborg Symbiosis, Denmark).

Despite the extent of cooperation in industrial symbiosis, there have been few attempts to create new industry from abundant industrial waste products. In the Kalundborg Symbiosis, bioindustrial WWs rich in nitrogen and phosphorous and other micronutrients can require multiple stages of treatment before discharge, which can add to the production cost. Meanwhile, the versatility of microalgae productions has the unique potential to close many waste loops in industries, which may valorize microalgae productions and financially incentivize industries to provide safe and viable nutrient waste streams (e.g. CO<sub>2</sub> flue gas and bioindustrial WW) in exchange for mitigated waste tipping fees and costly onsite treatments. Microalgae have long since been studied as a replacement for aerobic activated sludge removal of organic carbon and ammonium oxidation using photosynthetically produced oxygen which can offset the enormous costs of aeration (Craggs et al., 2011). Additionally, microalgae can assimilate unwanted WW nutrients and organic carbon in mixotrophic growth (Van Wagenen et al., 2015a). Within an industrial symbiosis, previously useless waste streams such as flue gases containing CO<sub>2</sub> can be used by microalgae for growth, while capturing CO<sub>2</sub> and possibly

valorizing the process further in a carbon credit economy by displacing fossil fuels (Campbell et al., 2011). Lastly, microalgae productions can also benefit from heat from the heated water and steam produced from nearby industry and cooled from non-potable water from neighboring WW treatment facilities.

An inherent drawback of the *ex post facto* approach of adding a microalgae production to an industrial symbiosis is that the nutrient rich WWs from industry may not comply with regulations for the gamut of products and markets created by a microalgae biorefinery. Similarly, these WWs may not be entirely effective at optimizing microalgae growth, whether it's from high salt concentrations (Podevin et al., 2017), abundance of deleterious chemicals, or because the WW stream does not contain the most preferred form of nitrogen for a given microalgal species (Podevin et al., 2015). Furthermore, WW fluctuations may also encumber microalgae product quality, as well. In practice, bioindustries often employ large treatment facilities to treat these waters after the residual biomass from the production is denatured at high temperature; however, these waters may still contain an array of chemicals that are not suitable for microalgal growth or human or animal consumption (W. E. Becker, 2013; Ehrenfeld and Gertler, 1997; Ehrenfeld and Chertow, 2003; Jacobsen, 2008). Incidentally, microalgae growth on industrial WW is most commonly employed for the removal of organic pollutants, heavy metals, biocides, and surfactants; where nitrogen and phosphorus removal is secondary to remediation of pollutants (Ahluwalia and Goyal, 2007; de-Bashan and Bashan, 2010; Mallick, 2002; Pittman et al., 2011). On the other hand, microalgae growth on agricultural and municipal WWs is mainly focused on nitrogen and phosphorus removal; however, producing high-value products (HVPs) for human consumption on this media is not a feasible approach from a regulatory standpoint. Therefore, alternative discharge infrastructures that can navigate around the introduction of harmful chemicals into waste streams and divert nutrient rich waters away from harmful chemicals may be required for an MBS from a regulatory and quality control standpoint. These diverted WW streams can be categorized under the broad term “technical waters,” which are waters or waste streams that can continue to be used for other applications after their initial use. Figure 4 shows the regulatory avenues for each possible byproduct or coproduct from microalgae in an MBS, whether the microalgae are grown from WWs, technical waters, or fertilizers. Ultimately, the work in this thesis aims to provide a framework for both WW producing bioindustries to consider microalgae productions as an alternative to

conventional WW treatment to financially incentivize industries to consider the industrially sustainable practices of an MBS.

In an MBS, microalgae bioproducts may be subject to myriad regulations, especially if industrial wastewaters (WWs) are to be used in a symbiosis. In the United States, it is generally accepted to adopt general manufacture practices (GMP) from “The Natural Products Quality Assurance Alliance” or “The Natural Nutritional Foods Association” among food industries; however, currently, the USFDA does not regulate food supplements and has been side stepped by the Natural Products Association (NPA), who now carry the regulatory torch. Though these regulations seem to be mostly self-guided in commercial applications, microalgae-derived pharmaceuticals in a biorefinery may change the regulatory climate by moving microalgae away from food supplements into drugs and pharmaceuticals. In most microalgae productions, it is generally assumed that toxins (e.g. environmentally accumulated metals) can be mitigated by proper cultivation techniques (W. E. Becker, 2013); however, this may not be the case in a microalgae biorefinery symbiosis (MBS) using industrial WW media with potentially harmful chemicals (Ahluwalia and Goyal, 2007; de-Bashan and Bashan, 2010; Mallick, 2002; Pittman et al., 2011). Currently, there are no standards for heavy metal concentrations of any microalgal products, with the exception of internal company guidelines (W. E. Becker, 2013). Moreover, as with maintaining product quality, real-time monitoring of microalgae productions with bioindustrial WW media can help mitigate regulatory concerns to valorize the MBS.

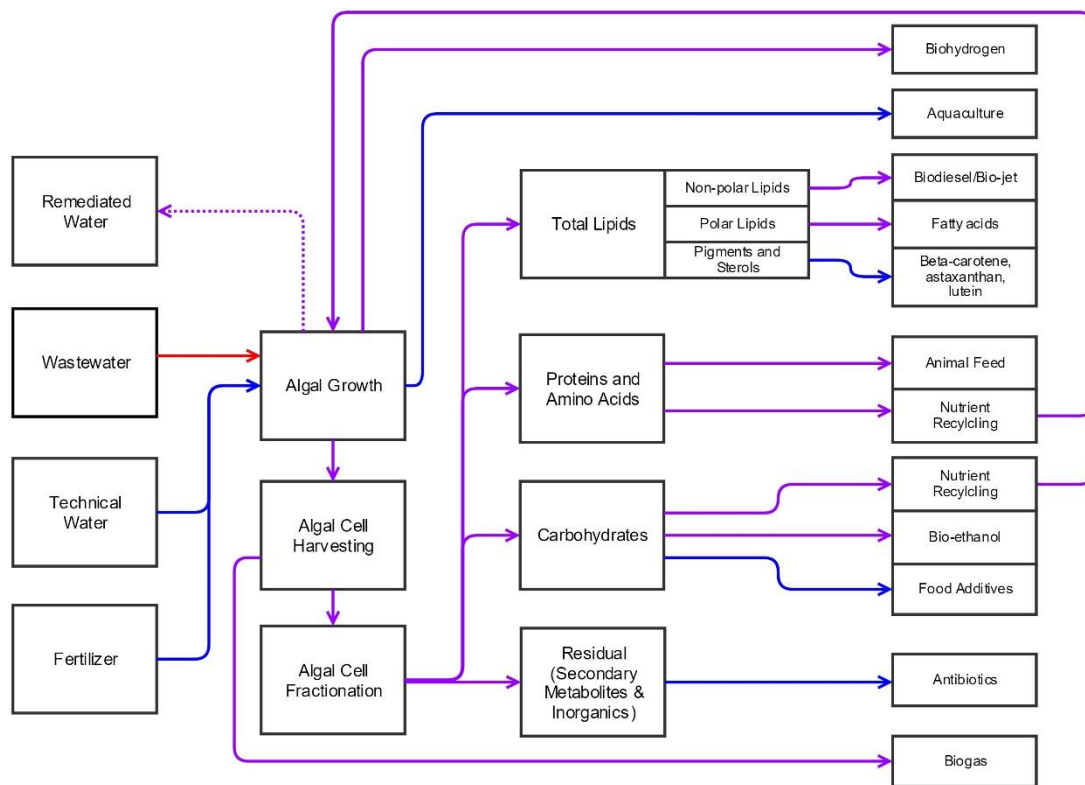


Figure 4. Bioindustrial WW industrial symbiosis scenarios for microalgae in a biorefinery. *Red lines* represent regulatory suspect WWs, either municipal or unregulated industrial waste streams: “dirty” water, *Blue lines* represent technical waters, industrial waters free from deleterious chemicals for human health: “clean water.” *Purple lines* represent either dirty or clean water. Figure adapted from Foley et al. (2011), Trivedi et al. (2015), and Koller et al. (2014).



### 3 Microalgae process analytical technology (PAT) and automation

Large-scale microalgae bioreactions are arguably one of the most difficult bioreactions to model and execute. In a biorefinery, routine monitoring of the feedstock composition is also important for valorizing the microalgae through fractionization (Ge, 2016) and ensuring HVP viability during extraction (Wijffels et al., 2010) and optimizing conversion processes for bioenergy (Chadwick et al., 2014). Altogether, these factors necessitate consistent product quality monitoring of these bioreactions. Monitoring of microalgal feedstock can be complicated for microalgae suspensions grown in outdoor PBRs exposed due to various conditions of light and temperature throughout the day, seasons, reactor inhomogeneities, in a variety of photobioreactor designs and configurations (Havlik et al., 2015; Posten, 2009). In more common bioreactions, such as bacterial and yeast fermentations, inhomogeneity is mostly limited to substrate and temperature gradients. However, in PBRs, gradients such as light, temperature, as well as self-shading microalgae cells further confound and complicate the system (Slegers et al., 2011), all which can change the quality of microalgal products at very short time scales (Bernard et al., 2015). In a microalgae biorefinery symbiosis (MBS), large fluctuations in inlet media composition may also occur, reiterating the need for more robust real-time monitoring to maintain a healthy culture and maintain product quality.

Due to the need for a thorough understanding of highly dynamic microalgal PBRs in a symbiosis amid the possible regulations and the need to monitor microalgal culture composition, quality, and purity in a biorefinery the commercialization of an MBS may benefit from bioprocess control and the guidelines set forth used both food and biopharmaceutical industries. In recent history, three movements have set forth guidelines to improve bioprocess control through time-resolute—real-time—monitoring of parameters and products; however, the movement has not yet been recognized for microalgae productions, which are still in their infancy. The United States Food and Drug Administration (USFDA) issued guidance for the bioindustries under the term process analytical technology (PAT) (U.S. Department of Health and Human Services Food and Drug Administration, 2004), European Medicines Agency's under the term "Quality by Design" (QbD) (European Medicines Agency, 2011), and the European Federation of Biotechnology (EFB) Section on Biochemical Engineering Science (ESBES) refers to a similar philosophy of



modeling, monitoring, measurement & control (M3C) (European Federation of Biotechnology, 2016; Luttmann et al., 2012). Moreover, due to the complexity of PBR systems, let alone a highly variable MBS with quality needs for multiple products, the MBS may have the most to gain from these widespread guidelines.

The most widespread guidelines—PAT guidelines—contain recommendations for routine monitoring of critical process parameters (CPPs), such as mixing/aeration/flow rate, pH, and temperature, while recommending the consideration of critical quality attributes (CQAs) (ICH, 2009). These CQAs are considered to be any physical, chemical, or biological parameters or attributes that can be measured to understand the quality of the product better. In microalgae productions, CQAs are considered to be the composition of lipids, proteins, carbohydrates, and HVPs for marketable products or for understanding the carbon allocation as a physiological status of the microalgae at various growth phases or in response to changes in substrates in the media or PBR environment. CQAs in microalgae productions can also be biological contaminants such as competitive microalga species, unwanted bacteria, or the physiological response of microalgae to chemical contaminants, as well. In the developing microalgae industry, various models have been created to improve the production efficiency of biomass and lipid synthesis; however, the lack of on-line PAT to model the complex reactions in PBRs from light and temperature fluctuations is very limited (Bernard et al., 2015), which is even more limited in an MBS. Furthermore, in the interest of an MBS, the need for a cost-effective, high-selectivity, real-time monitoring tool for various microalgal components becomes increasingly necessary for addressing the quality and regulatory criteria.

As discussed in section 1.2, in an outdoor MBS using bioindustrial WW, standard models may not be suitable for controlling the productivity of microalgal biomass and biosynthesis of HVPs alone. Model predictive control (MPC), or multivariate process control (MVPC) uses advanced non-linear models to create a dynamic feedback of bioprocesses, essentially creating a fingerprint of the reaction from high time-resolution data, or “big data” (paper III), which can then be used to automate the microalgae production. A popular MVPC tool, which relies on big data—artificial neural networks—has already made its way into the field of microalgae biotechnology to monitor and automate bioreactions (Hu et al., 2008; Jung and Lee, 2008). The evolution of MVPC can also improve software sensors, or soft sensors, which are capable of correlating real-time data from standard CPPs (e.g. pH) with infrequent or frequent off-

line analysis of CQAs (lipids, carbohydrates, proteins etc.). These soft sensor models are used to interpolate or infer intermediate values of a CQA by relating them to patterns found in the CPPs, effectively creating a software based, virtual, real-time analysis of CQAs (Arranz et al., 2008; Baughman and Liu, 1995; Cecil and Kozłowska, 2010; del Rio-Chanona et al., 2016; Faassen and Hitzmann, 2015; García-Camacho et al., 2016; Havlik et al., 2015; Jung and Lee, 2008; Khataee et al., 2010; Komives and Parker, 2003; López-Rosales et al., 2013; Maier and Dandy, 2001; Millie et al., 2012; Podder and Majumder, 2016; Sharon Mano Pappu et al., 2013; Takahashi et al., 2015; Yoo et al., 2016, 2015) (paper III). Improvements in computational chemometrics have also paved the way for computational inferences of parameters and molecules that are not directly measured (e.g. photosynthetic efficiency) to improve monitoring of microalgae in a biorefinery (paper III). With vibrational spectroscopy, real-time CPPs, as well as real-time CQAs, have the possibility to aid in the creation of very robust MVPC models and software sensor models for a wide range of molecules in microalgal cultures, with the possibility of monitoring microalgal response to changes in WW media and PBR environment (paper III). These advanced tools may also negate the need for the permanent use of immersed probes, which may circumvent contamination concerns. From the quality concerns of a biorefinery to the complexity of an outdoor PBR producing microalgae grown on fluctuating WW media, and the overall valorization of these processes in the midst of regulations, an MBS may critically benefit from real-time monitoring of CQAs under the PAT guidelines.



## 4 Screening of microalgae

As an initial step to expedite microalgae productions in a microalgae biorefinery symbiosis (MBS), high-throughput and time-resolute microalgae screening can be a very effective tool to analyze the growth response of variety microalgal strains to various types and concentrations of wastewater (WW) media, as well as to optimize growth media, in general. For microalgae productions using dynamic WW media for growth, there is the possibility that these WWs may have varying proportions of nitrogen species (nitrate, ammonium, and urea), in which the presence of one may inhibit the use of another (Podevin et al., 2015). Therefore, there is also need to screen for robust species capable of enduring these fluctuations. Overall, the diversity of potential waste or technical water streams, as well as the vast diversity of potential microalgae species and mutants, makes large-scale screening efforts a daunting and challenging task (Van Wageningen et al., 2014).

Currently, small-scale screenings usually performed in Erlenmeyer flasks—shake flasks—where samples are often physically extracted for analysis, are analytically limited by the amount of sample volume that can be extracted from the flask when measuring specific growth rates (SGRs). As samples are extracted, mixing properties and even light availability can change depending on the experimental setup, which can influence the growth of microalgae. It follows that as more time-resolute measurements are needed, these experiments require more sample volume and subsequently larger sample containers, larger experimental setups and laboratory spaces, and ultimately more person-hours. Some authors suggest that fluorescence can be used to *observe* microalgae growth in microplate readers, and mention the possibility of measuring growth rates with light absorbance in them (Sieracki et al., 2005); however, there are very few demonstrations of fluorescence *growth rate* detection inside microplates (Fai et al., 2007; Skjelbred et al., 2012). In paper I, microalgae were grown in batch reactions inside 24-well microplates, where SGRs were determined *in vivo* using chlorophyll-a autofluorescence of microalgae cells measured in a microplate reader, without any wasted sample. With footprints around 100 cm<sup>2</sup> for 12 to 96 well samples and scanning times of 1 to 2 minutes, the high-throughput microplate method can reduce the laboratory footprint of screenings and the analysis time compared to shake flask screening assemblies and experiments significantly.

Apart from the practical high-throughput limitations of shake flask screenings, physiochemical changes can arise when screening microalgae on WWs or various concentrations of different nitrogen sources. The most pronounced physiochemical change in WW and nitrogen source screenings are the changes in pH during nutrient assimilation. Batch cultivations of microalgae containing ammonium as a nitrogen source (common to WW) can experience a substantial drop in pH due to the consumption of bicarbonate associated with ammonium assimilation (Grobbelaar, 2013; Mayer et al., 1997; Podevin et al., 2015). On the other hand, during nitrate assimilation in batch cultures, pH can increase due to the production of bicarbonate (Grobbelaar, 2013). Given these effects, batch culture screenings are vulnerable to the influence of these pH changes and may give false negatives and positives for the growth of potential WW media that cannot adequately buffer these changes in pH, which can otherwise be buffered at industrial scales, at a cost. Moreover, the pH change from the assimilation of a nitrogen source does not reflect a microalga species' preference or aversion for that nitrogen source, but simply reflects the effect of pH on growth. Similarly, in shake flask experiments that rely on dense microalgal samples for cell detection (e.g. dry weight), the increasing concentration of cells during growth can influence the availability of light reaching microalgae cells hindering photosynthesis and influencing the SGR (Slocombe and Benemann, n.d.). Research in paper I highlights that by detecting the microalgae at low concentrations—low detection—with *in vivo* chlorophyll-a autofluorescence, the SGR of the microalgae for each nitrogen source can be ascertained regardless of nitrogen source before large physiochemical changes or significant self-shading occur.

Thus, the combined benefits of high-throughput and low detection of microalgae have led to the development of the microplate screening method described in paper I. This screening method utilized the low detection of the fluorescence signal of chlorophyll-a using small footprint 24-well microplates analyzed in a Synergy Mx multimode microplate reader (BioTek Instruments, Inc., USA). In paper I, by comparing buffered ammonium carbonate media and unbuffered ammonium media in samples extracted from shake flasks and analyzed with the fluorescence method it was initially established that the SGR of the green microalgae *Auxenochlorella protothecoides* could be determined before the pH drop from ammonium assimilation occurred (Figure 5). The SGRs of the flask cultures grown on ammonium carbonate and ammonium chloride were  $0.88 \pm 0.03$  and  $0.93 \pm 0.10 \text{ day}^{-1}$ , respectively; however, there

was no statistically significant difference between SGRs before the pH drop in ammonium media occurred ( $P = 0.543$ ).

The work also demonstrated that the correlation of the fluorescence signal expressed as relative fluorescence units (RFU) vs. cell number (Coulter cell count) began to taper off in a second order polynomial trend at higher RFUs and cell numbers (Figure 6). This gradual drop in the trend line may be described by the gradual shading of the fluorescence signal by neighboring microalgal cells. In figure 5, the difference between *Chlorella Vulgaris* and *Nannochloropsis oculata* trend lines may be because of the relative abundance of the chlorophyll-a in each microalgae species. It is evident that the curve “drops” at different RFU for each microalga species, which may depend on the content of chlorophyll-a per cell and the total number of cells in the sample. Therefore, the drop is likely not related to a limitation of the microplate reader detection instrumentation and is more likely a physiological property (e.g. chlorophyll concentration and self-shading) native to each microalga species. The same effect was also observed in *Auxenochlorella protothecoides* and *Chlorella sorokiniana* (unpublished). It is expected that changes in chlorophyll concentration in each microalgae cell will depend on the amount of irradiance used in the experiment, in which the amount of chlorophyll can increase in cells at lower light intensities (Neidhardt et al., 1998). In all cases of the microalgae grown under  $60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  in this experiment, microalgal cell fluorescence and the corresponding SGR could be measured before the correlation became non-linear, which was best fit as a second order polynomial curve ( $>15,000$  RFU). The polynomial drop of RFU can be observed in both Figure 6 and Figure 7. Therefore, under the conditions of this experiment detailed in paper I,  $60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  was enough light to promote enough chlorophyll synthesis for fluorescence detection, while also making this method more streamlined since a direct linear correlation of the four microalgae to cell number can be made at this light intensity. The direct linear correlation is not only mathematically convenient but, moreover, there is no need to produce a correlation of actual cell number to RFU specific to each microalga species in a given condition. This knowledge can be used to avoid the need for cell counting and ultimately reducing analytical machinery down to just a microplate reader.

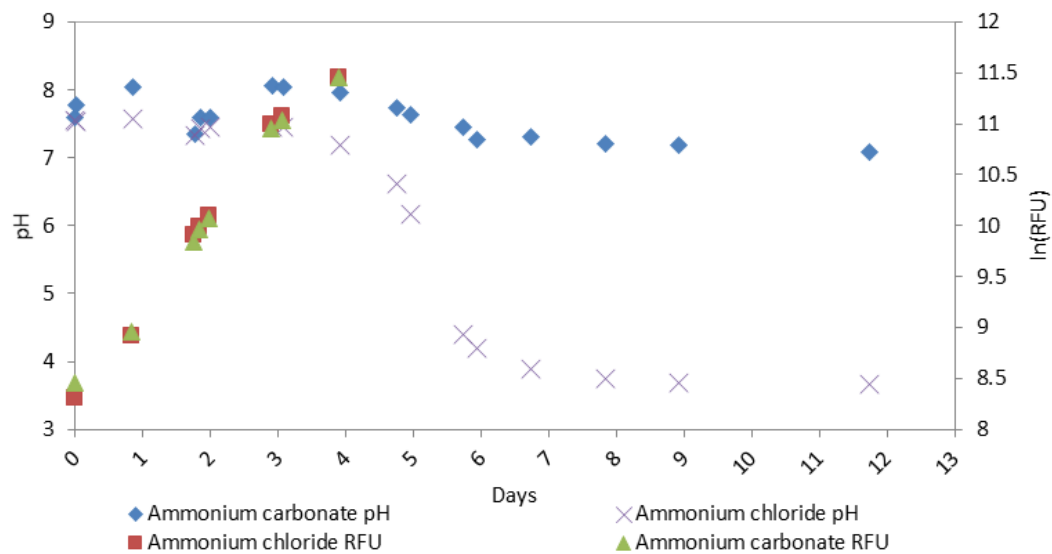


Figure 5. Time course pH and the natural logarithm of the fluorescence (RFU) of *Auxenochlorella protothecoides* grown in ammonium carbonate (buffered) and ammonium chloride (unbuffered) media. A sharp drop in unbuffered ammonium chloride media can be observed; however, the SGR can be determined before the pH drop occurs.

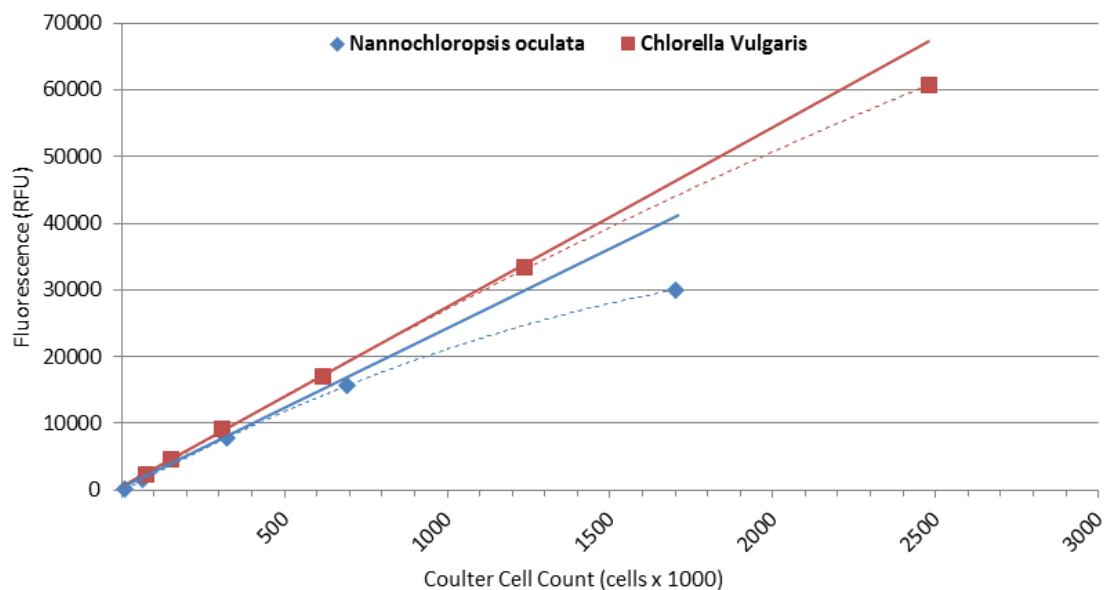


Figure 6. Fluorescence (RFU) vs. Coulter cell number for *Chlorella vulgaris* and *Nannochloropsis oculata*. The solid red line and solid blue line represent the linear regression of the first five data points of *C. vulgaris* and *N. oculata*, respectively. The curved dotted lines represent the second-order polynomial regression trend for each algae species and possible cell shading of the fluorescence signal at higher cell densities and chlorophyll concentrations.

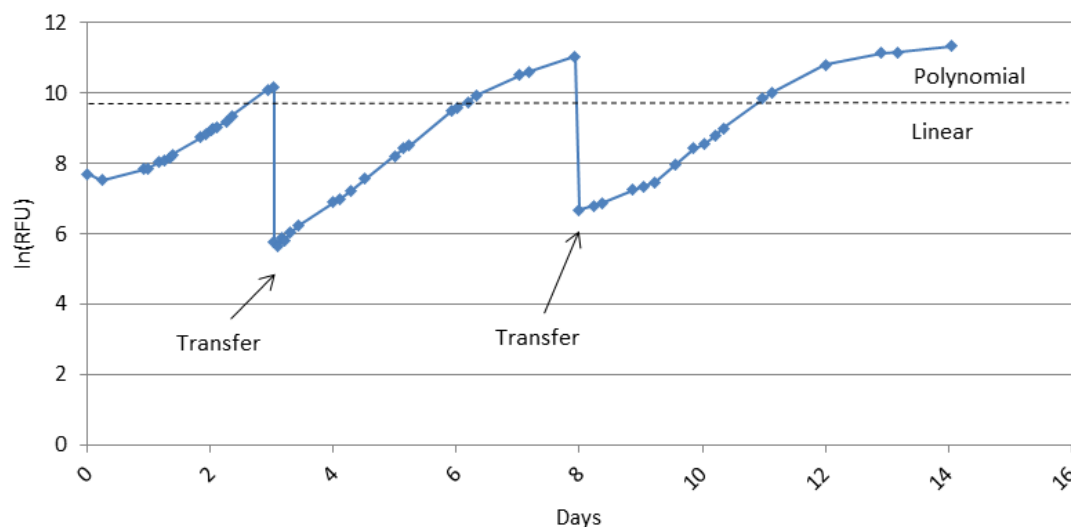


Figure 7. The natural logarithm of fluorescence (RFU) of *N. oculata* cultivated in media containing ammonium chloride over two serial transfers (arrows). During all growth cycles, biomass and subsequently SGR could be determined before 15,000 RFU (above dashed line). The microalgae were cultivated until the upper fluorescence limit was reached in the last transfer.

The developed microplate assay was then used to test SGRs of all four microalgae on four different nitrogen sources: ammonium carbonate, ammonium chloride, sodium nitrate, and urea (Figure 8). Serial transfers of the microalgae were performed to facilitate three cycles of growth for acclimation of the microalgae to the media. The SGR of *C. vulgaris* showed significantly ( $P < 0.05$ ) higher SGRs when grown nitrate, compared to the other nitrogen sources. As expected, *A. protothecoides* did not grow on nitrate, with significantly higher growth rates in ammonium carbonate grown microalgae compared with ammonium chloride (paper I). During the second and third generation, after acclimation, *C. sorokiniana* showed no significant difference between any of the nitrogen sources. Similarly, after omitting the first generation of growth of *N. oculata*, the microalgae showed no significant difference in SGR for the microalgae grown on ammonium carbonate, ammonium chloride, and urea, with a clear aversion for nitrate. In general, *C. sorokiniana* was the most resilient to the effects of adjustment in the first generation. *N. oculata* was also resilient to these effects except for the case of urea. The results also showed that *N. oculata* was a viable model organism with very little data variance using this method. In paper I, it was demonstrated that there was no significant difference between the SGRs of *C. vulgaris*, *A. protothecoides*, *C. sorokiniana*, and *N. oculata* grown on ammonium chloride and ammonium carbonate reiterating



that the SGRs for all species grown on ammonium could be determined before large pH changes occurred.

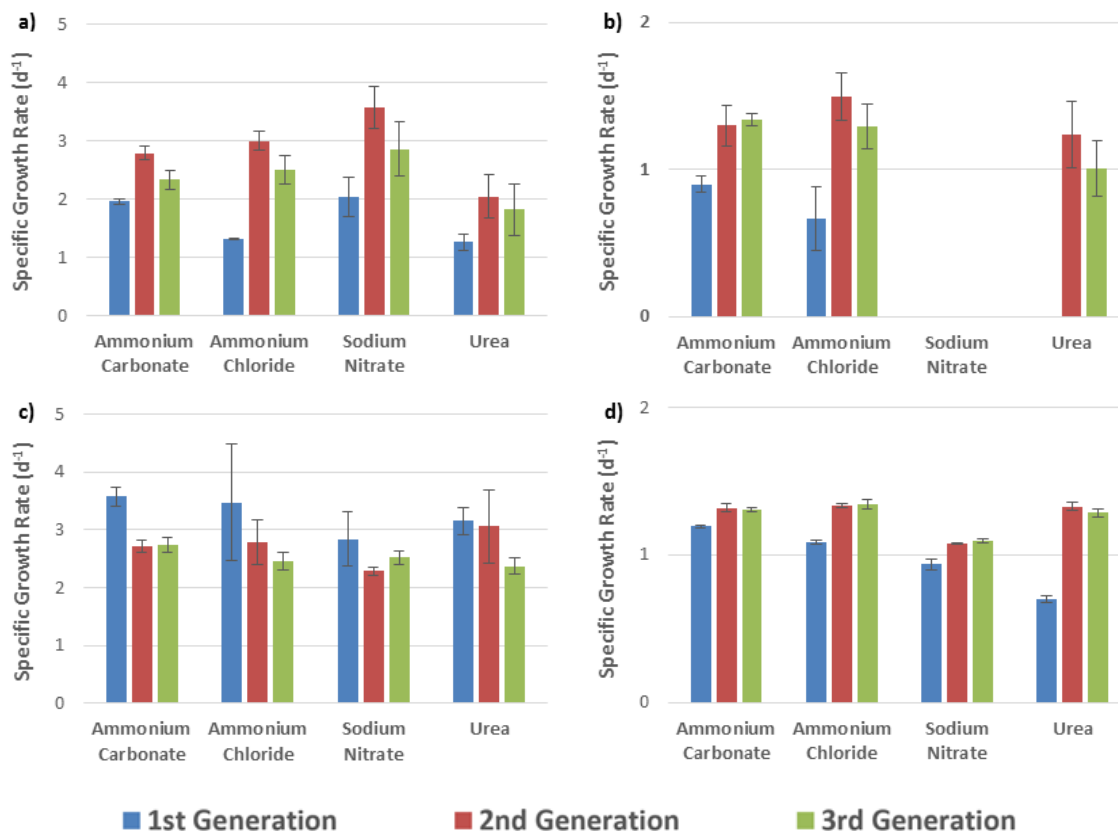


Figure 8. SGR ( $\text{day}^{-1}$ ) of a) *Chlorella vulgaris*, b) *Auxenochlorella protothecoides*, c) *Chlorella sorokiniana*, and d) *Nannochloropsis oculata* grown over three cultivations on the four different nitrogen sources (ammonium carbonate, ammonium chloride, sodium nitrate, and urea). The nitrogen source concentration was 1 M nitrogen. Note the *different scales* of the y-axis.

The combined benefits of providing an accurate SGR before nitrogen assimilation pH changes and self-shading occur make *in vivo* early-detection of chlorophyll-a autofluorescence in microplates a very promising high-throughput technology for WW screening. In paper I, it was demonstrated that there were notable differences in microalgae SGRs when grown on different nitrogen sources as well as during the acclimation period of the microalgae to new media. The ease of producing three generations of data in microplates make this method more conducive to scientific accuracy by properly adapting the microalgae to new environments before screening, but also elucidates the ability of microalgae to adapt to new media. For microalgae grown on WW, the ability of a microalgae to adapt from variations in nutrient type, concentration, and

proportions can be better understood by quickly observing acclimatization in microplates, which cannot easily be executed in shake flasks. Not only are microplates spatially high-throughput, allowing for vastly more samples than shake flasks in a given area, the low and early detection of growth through autofluorescence also make this method temporally high-throughput, measuring SGRs of microalgae at a fraction of the time of optical density methods in larger vessels and even the same microplate vessel (unpublished). The screening method can also be used to monitor SGR responses to irradiance and optimal dilutions of WW in the format described in paper I. The high-throughput turnover of this method as well as the non-obvious benefits of scaling down for low and early detection through fluorescence spectroscopy make this a promising screening alternative to shake flasks towards quickly identifying viable microalgae species for an MBS.



# 5 Full-scale PBR microalgae production

## on bioindustrial wastewater

### 5.1 Photobioreactor design

Two critical factors determining valorization of a microalgae biorefinery symbiosis (MBS) are the areal productivity and the cost of a photobioreactor (PBR). Microalgal PBRs come in a variety of shapes, areal footprints, configurations (batch, plug flow, or continuous operations), however, of the pilot and large-scale studies in existence today, most reactors are ultimately designed around the species of microalgae, the media (saline, fresh, wastewater), latitude, and weather. In a given environment, there are numerous ways to ensure that microalgae are receiving the proper amount of light. Either too much (photoinhibition) or too little light can be problematic for microalgae productions. The same is true for temperature, which is highly interrelated with light status when it comes to microalgae growth efficiency (Coles and Jones, 2000; Huner et al., 1998; Maxwell et al., 1995). The two main types of light control are temporal light dilution and spatial light dilution. The former, commonly known as “the flashing light effect,” relies on mixing to transport microalgae between zones of low and high irradiance, depending on the culture density (Abu-Ghosh et al., 2015; Degen et al., 2001; Kok, 1956). Spatial light dilution relies on the orientation of the reactor, where the reactor is oriented to receive indirect, diffuse sunlight to avoid photoinhibition (Chini Zittelli et al., 2006; Tredici and Zittelli, 1998; Zittelli et al., 2013). PBR designs relying on diffuse light from spatial light dilution are situated in rows of panels or stacked tubes receiving diffuse light from above. In this format, the reactor height and width between rows is optimized to receive ample dilute light as the position of the sun changes (Slegers et al., 2011; Wijffels and Barbosa, 2010). The Ecoduna (Ecoduna, Austria) “solar-tracking” PBR discussed in paper II was designed to use diffuse light in spatial light dilution format, while simultaneously receiving direct light by mechanically tracking the sun throughout the day essentially creating an artificial solar noon, with sunlight reaching between each equally. The panels can also be situated with a small angled offset allowing more or less direct sunlight to reach the panel (Figure 9). The six-meter tall

modules of this system are placed in rows that are positioned to optimize light between each module for the location in Kalundborg, Denmark ( $55.6864^{\circ}$  N,  $11.0892^{\circ}$  E). Ultimately, this solar tracking design has fewer light limitations than a stationary light-dilution format, which was designed to increase areal productivity of a microalgal production to valorize this PBR.

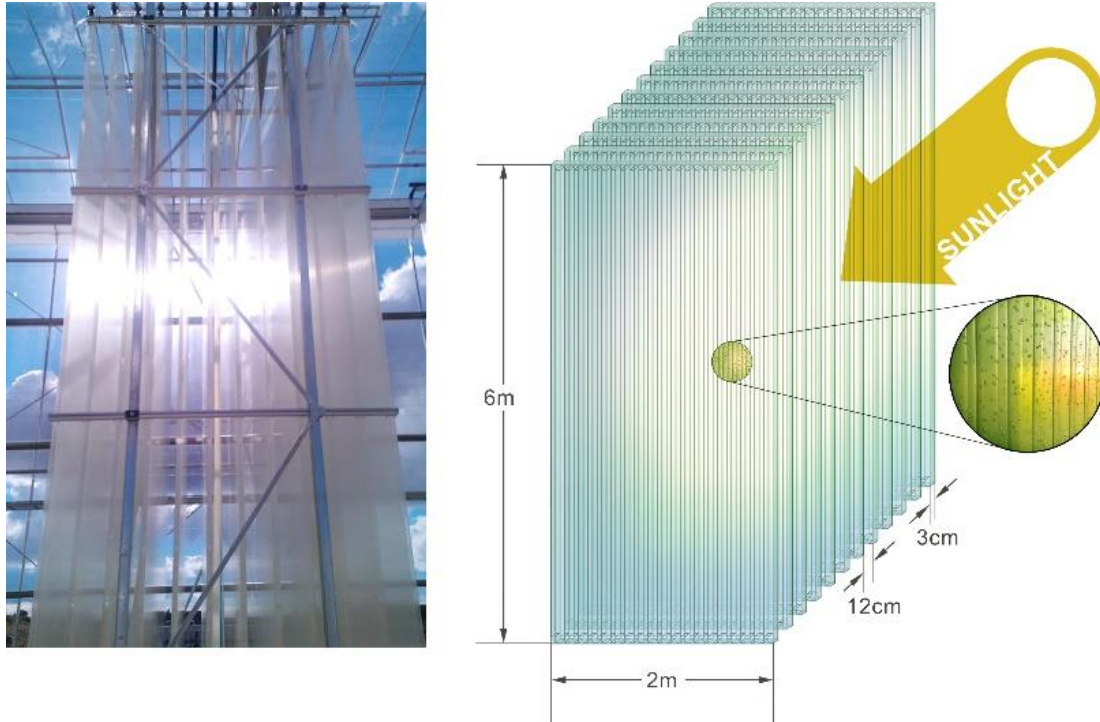


Figure 9. Photo of light dilution design in Ecoduna PBR, where each panel can be seen receiving direct sunlight (*left*), design schematic of Ecoduna PBR with dimensions; *callout* demonstrating microalgal growth, aeration bubbles, and the effect of sunlight reaching the panel (*right*). The arrow represents direct sunlight reaching one face of every panel as the module tracks sunlight.

## 5.2 Bioindustrial wastewater composition and pre-treatment

In a given bioindustry, there are several waste streams with nutrients from spent biological cells that can be considered for microalgal growth. In the Novozymes (Novo Nordisk, Denmark) bioindustry, before wastewater (WW) is discharged, the biological content of the water from insulin and enzyme productions is thermally deactivated by intense heating. This step may be crucial

in valorizing its use in microalgae productions as a growth media since this water is sterilized, which is beneficial for maintaining contaminant free monocultures. However, it is unknown whether these waters contain compounds harmful to microalgae growth but corporate efforts could be made to minimize contaminants (e.g. surfactant and disinfectants) in these bioindustrial processes. After that, the thermally deactivated WW (WW influent) is treated, and a biologically dense reject water (WW treatment reject water) is separated from the treated WW effluent. Reject water can then be treated by an onsite internal circulation (IC) anaerobic digestion (IC effluent). The flow rate and compositions of each waste stream can be seen in Table 1. In fact, Novo IC WW has been studied as a microalgal growth medium (Van Wagenen et al., 2015b, 2014). For the study in paper II, treated Novozymes WW effluent was chosen over other waste streams as a precaution against contaminants, which may otherwise be treated and removed. This choice also avoided the high solids content expected in the WW influent after deactivation of cells, which would be undesirable for pre-treatment with ultrafiltration before the microalgal production. Despite having lower nutritional content than the other WWs, the treated WW effluent likely contained the least amount of unknown harmful agents, which is more suitable for freshwater microalgae growth. For example, the IC digestion uses sludges from the original water treatment step, which could contain accumulated harmful agents released during digestion. Similarly, despite the relatively low amount of nitrogen compared to the IC WW stream, it was decided that IC WW was a poor representation of other bioindustries that are not equipped with anaerobic digestion.

Table 1. Nutrient characteristics of potential bioindustrial WW waste streams from Novozymes, in which WW effluent (*gray box*) was used for microalgal growth in the PBR. The average, peak and the standard deviation of the average are shown for each water.

	WW Influent			WW Effluent			WW Treatment Reject Water			IC Effluent		
	Avg.	Peak	StD	Avg.	Peak	StD	Avg.	Peak	StD	Avg.	Peak	StD
Flow rate (m <sup>3</sup> d <sup>-1</sup> )	6788	10633	1293	8554	12572	1291	8554	12572	1291	4303	8380	2543
Total Nitrogen (mg L <sup>-1</sup> )	401	1754	143	22.0	38	4	766	4290	301	435	571	90
Ammonium (mg L <sup>-1</sup> )	-	-	-	9.2	21	2	-	-	-	292	486	49
Nitrate (mg L <sup>-1</sup> )	-	-	-	1.6	4	0	-	-	-	-	-	-
Total Phosphorus (mg L <sup>-1</sup> )	43	198	33	2.8	8	1	79	247	36	-	-	-
Ortho-phosphate (mg L <sup>-1</sup> )	-	-	-	1.5	4	45	-	-	-	-	-	-
VFA (mg L <sup>-1</sup> )	-	-	-	-	-	-	-	-	-	318	1081	254
COD (mg L <sup>-1</sup> )	6913	11894	1625	216.8	408	45	8501	15363	2178	2569	5097	900
Suspended Solids (mg L <sup>-1</sup> )	1	58	5	68.6	171	32	-	-	-	-	-	-

With WW effluent as a growth media, the treated WW effluent contained unsettled biologically active bacterial biomass, which would also need to be removed before the microalgae production. Following the acquisition of Novozymes WW effluent media, an industrial ultrafiltration system (LiqTech International A/S, Denmark) was used to remove microorganisms, solids, and debris before using the media for microalgal growth, which can interfere with mixotrophic microalgal growth and propagate contamination by assimilating organic carbon present in the Novozymes WW effluent media ( $\text{COD} = 230 \text{ mg L}^{-1}$ ). In paper II, the  $0.4 \text{ }\mu\text{m}$  ultrafiltration of the Novozymes WW effluent media was reported to reduce coliform, e-coli, and colony forming units by 99.88, 99.59, and 99.97, respectively. The nutrient content in the WW media after ultrafiltration remained near the average for TN ( $22 \text{ mg L}^{-1}$ ) and above average for ammonium ( $10 \text{ mg L}^{-1}$ ) and orthophosphate ( $3 \text{ mg L}^{-1}$ ). As expected, there was no decrease in sodium ( $1300 \text{ mg L}^{-1}$ ) and chloride ( $1100 \text{ mg L}^{-1}$ ) concentrations during filtration, which remained in the WW media (paper II).

## 5.3 Microalgae growth on bioindustrial wastewater

In paper II, the versatile microalgae *C. sorokiniana* was grown on defined and bioindustrial WW media (Novozymes WW effluent) at sub-optimal irradiance and temperature in the novel PBR discussed in 5.1. As discussed in section 4, the high SGR, and demonstrated the ability to easily acclimatize to new nitrogen sources, mixotrophy, and known resilience to extreme conditions, make *C. sorokiniana* a viable candidate to grow on WW containing several nitrogen sources. Immediately following inoculation of the bioindustrial WW with the microalgae, exponential growth was observed. Following inoculation, only  $6.27 \text{ mg NH}_4^+\text{-N L}^{-1}$  of the original  $10 \text{ mg NH}_4^+\text{-N L}^{-1}$  remained in the WW media; however, the SGR for the four-day period until all ammonium was consumed was  $0.48 \text{ day}^{-1}$ . During this period, it was demonstrated that there were no noteworthy adverse effects of the WW on *C. sorokiniana* growth despite high concentrations of sodium ( $1300 \text{ mg L}^{-1}$ ), chloride ( $1100 \text{ mg L}^{-1}$ ), and sulfate ( $700 \text{ mg L}^{-1}$ ) and despite the low concentrations of zinc and manganese, which were later supplemented with Compo nutrient solution. Furthermore, it was shown that growth was obtainable at low temperatures and low solar insolation on ammonium. A time series representation of  $\text{OD}_{735}$  changes with temperature and solar insolation can be seen in Figure 10.



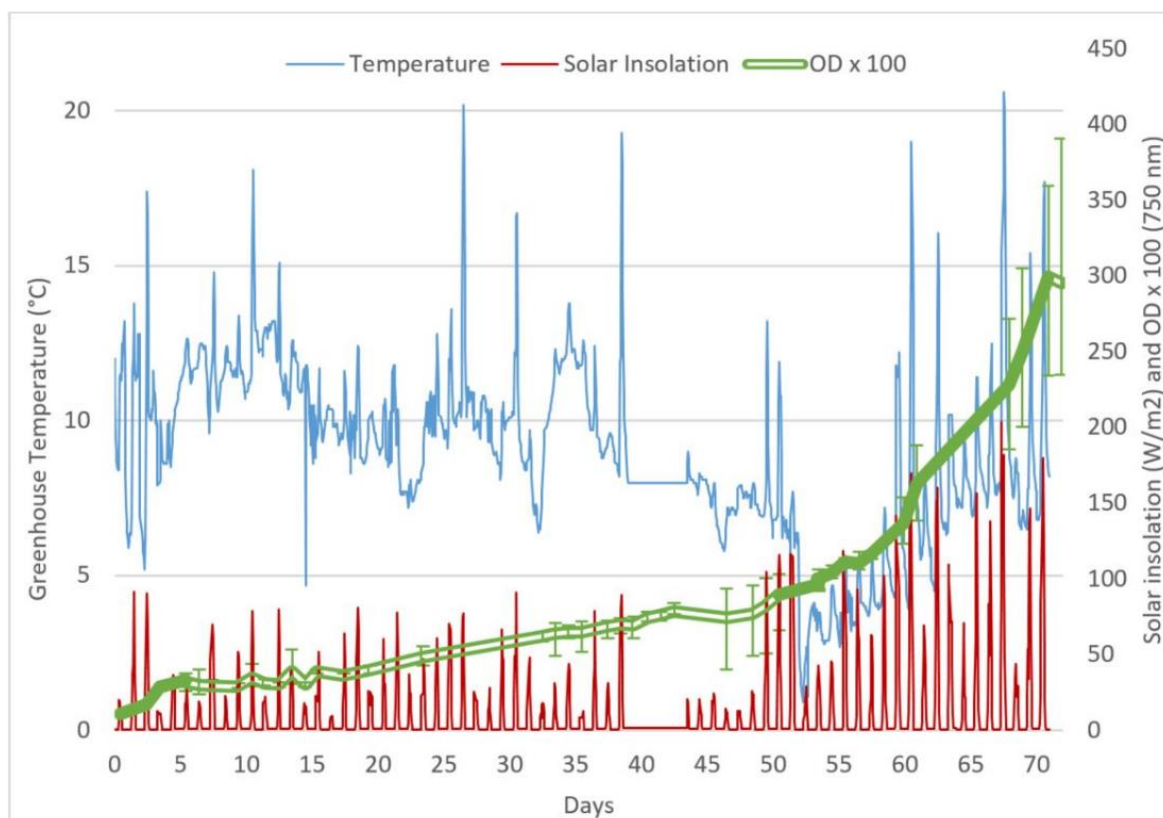


Figure 10. Paper I, assay-II time series daily greenhouse temperature ( $^{\circ}\text{C}$ ; blue), solar insolation ( $\text{W m}^{-2}$ ; red), and the mean optical density at 735 nm ( $\text{OD}_{735}$ ; green) of three reactor panels.  $\text{OD}_{735}$  has been scaled ( $\text{OD}_{735} \times 100$ ) to y-axis on the right. The solid green line for  $\text{OD}_{735}$  represents data used to calculate predominant SGRs, compared to hollow green lines, which were not used in growth calculations. Bars above and below the time series  $\text{OD}_{735}$  represent the maximum and minimum  $\text{OD}_{735}$  values of the three reactor panels.

After the four days of growth on ammonium, the production was continued by supplying the reactor with  $123 \text{ mg NO}_3\text{-N L}^{-1}$  and  $16.96 \text{ mg PO}_4\text{-P L}^{-1}$  and 1.5 L of Compo nutrient solution to see if the bioindustrial WW had any long term adverse effects on the production. A surprising result was that the transition was followed by a long lag phase from days 5 to 51 (Figure 10), despite not having any nutrient limitations. Though in paper I it was demonstrated that *C. sorokiniana* was a viable production species because of its swift transition between nitrogen metabolisms, at large-scales with low irradiance and low temperature, the adverse effects of a metabolic shift may be more pronounced. It is possible that the down-regulation of nitrate reductase in *C. sorokiniana* was caused by the presence of ammonium (Bates, 1976; Terry, 1982). Furthermore, nitrate assimilation is largely mediated by light (Stöhr et al., 1995), whereas ammonium assimilation is not. These combined factors may have shifted the microalgae into a dormancy ending on day 52 when the culture transitioned

into a predominant production phase with an SGR of  $0.15 \text{ day}^{-1}$ . Incidentally, this occurred after a surge in solar insolation and temperature and despite the drop in both temperature and solar insolation in the following days, growth continued. Despite being mesophilic, *C. sorokiniana*, with a minimum temperature to sustain growth of  $5.2^{\circ}\text{C}$  (Sorokin and Krauss, 1962), could sustain growth after the greenhouse temperature reached as low as  $4.2^{\circ}\text{C}$ .

In paper II, it was demonstrated that microalgae grown on bioindustrial WW outperformed the same microalgae grown on defined media. The results showed that the microalgae grown on the Novozymes WW effluent (assay II in paper II) have more than a 35% higher SGR than the microalgae grown on freshwater microalgal media (assay I in paper II), during the predominant production phases of each assay. Despite having lower temperatures and lower solar insolation in assay II than in assay I, it is expected that the higher growth rate was due to the presence of organic carbon in the WW of assay I, which was not present in assay I. The decreasing in chemical oxygen demand, as a proxy for organic carbon from  $230 \text{ mg L}^{-1}$  to  $30.25 \text{ mg L}^{-1}$ , reflects that the microalgae may have assimilated organic carbon. Alternatively, the microalgae in assay II may have also benefitted from the coupling of lower solar insolation and lower temperatures, in which a coupling of high light and low temperatures can result in high photosystem II excitation pressures, decreasing photosynthetic efficiency (Huner et al., 1998; Maxwell et al., 1995). Ultimately, it can clearly be stated that microalgae can grow on Novozymes WW effluent, and likely on a number of other bioindustrial WW sources towards facilitating an MBS. However, in order to have a thorough academic understanding of the multitude of reactions occurring at fluctuating temperatures and solar insolation, there is a need for time-resolute monitoring of these reactions to make concrete conclusions about these complex assimilation and growth mechanisms.

## 5.4 Harvesting with microfiltration

For decades, there have been many reported methods to harvest colloidal microalgal cells cheaply. In a biorefinery of microalgal cells, the challenge of cheap harvesting remains amid the new engineering concerns of ensuring the viability of microalgae and their bioproducts during harvesting. In paper II, after two productions of microalgae, a  $1.0 \mu\text{m}$  microfiltration unit was tested to simulate the upconcentration of the microalgae as an initial harvesting step in a biorefinery setting. During microfiltration, upconcentrated microalgae exhibited a change in cell viability as more and more cells were exposed to the

microfilter. During the microfiltration process, microalgae interact with the ceramic microfilter membrane, and the upconcentrated microalgae return to the container until the supernatant is removed. During this process, the container was shaken and samples were taken for 1000 L (unfiltered), 750 L, 500 L, and 250 L and the viability of these cells at each volume of concentrated microalgal suspension was tested with fluorochrome staining. Initially, the unfiltered microalgae suspension contained 1.81 % dead cells. At the end of microfiltration, at 250 L of condensate, approximately 20.7% of the cells died during microfiltration, with a relationship of cell death and condensate volume shown in Figure 11. Similar relationships have been observed in literature with research focused on the release of TOC and the accumulation and fouling of EPS on filter membranes (Babel and Takizawa, 2010; Ladner et al., 2010; Wicaksana et al., 2012); however, the relationship to cell death of *C. sorokiniana* may better depict the potential of losing high-value products (HVPs) during the rupturing of microalgae cells. Moreover, rupturing cells and the avoidable loss of HVP is not desirable for to valorize microalgal productions within an MBS.

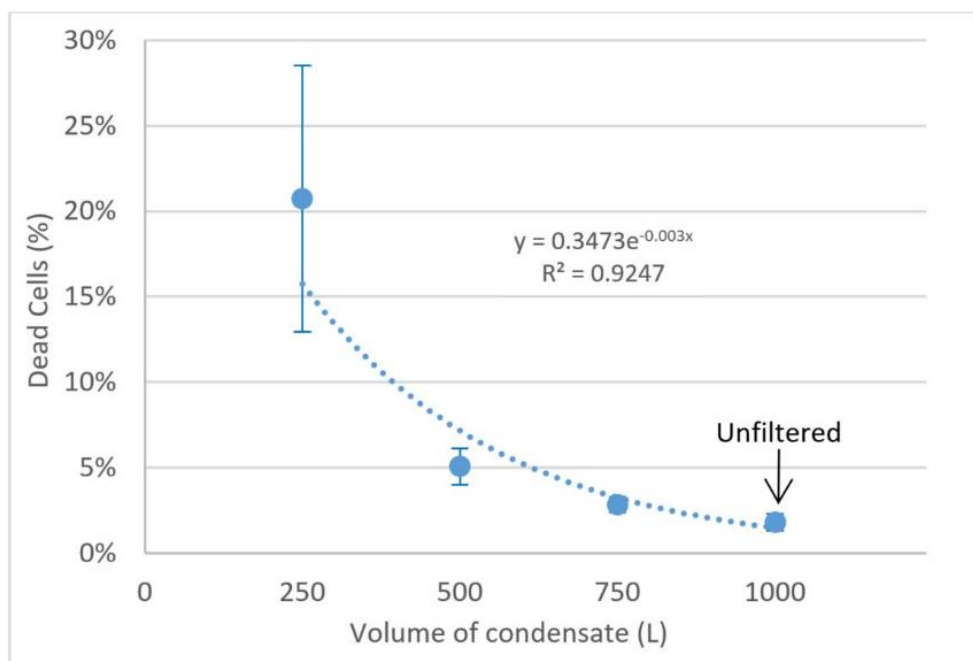


Figure 11. Exponential loss of cell viability after cultivation (unfiltered) and diminishing volumes of microfiltration condensate. Data points are the mean percentage of dead cells and duplicate range (bars).

## 6 Towards modeling complex large-scale photobioreactors

From understanding the nuances of microalgal growth at microplates scales to observing the complicated dynamics of a large-scale, outdoor photobioreactor (PBR), the benefits of real-time monitoring of these highly dynamic systems for a microalgae biorefinery symbiosis (MBS) are increasingly obvious. With the outlook of microalgae productions moving towards a biorefinery, high-selectivity on-line/in-line monitoring might not only benefit the bioprocess productivity but also be critical for quality control of microalgae products directed at various markets. Of the available on-line and in-line monitoring tools today, only a few have the ability to elucidate information of microalgae growth, the multitude of products in microalgae to be extracted in a biorefinery, contaminants, and even some critical process parameters (CPPs). Unlike highly controlled screening studies, large-scale, outdoor reactions are perceptible to a multitude of inconsistencies and inhomogeneities. Basic assumptions can be made about a system. However, the multitude of nuanced enzymatic relationships of microalgae production to temperature and light make these systems difficult to understand when temperature and light gradients exist daily, which can fluctuate at small time scales (Bernard et al., 2015). These challenges are further confounded for microalgae using WWs with varying types, concentrations, and proportions of both nutrients and adverse chemicals.

Not only is there a need to monitor microalgae bioreactions in real-time, but it may also be important to monitor several reactor locations depending on the design of the reactor, residence times, and mode of operation to understand the operation fully. Spatial gradients of pH, dissolved oxygen, CO<sub>2</sub>, and light are known to occur in low-tech, high rate algal ponds (HRAPs) over very large surface areas (Havlik et al., 2016). In paper II it was demonstrated that over the course of the five-kilometer-long, closed, recirculating plug flow reactor in the Ecoduna PBR module, time-series optical density measurements began to diverge depending on the location of measurement possibly due to inhomogeneities propagated by anisotropy. Figure 12 shows that at 57 days into the experiment, at the beginning of the predominant production phase of the experiment discussed in section 5.3, the concentration of microalgae cells inside the reactor at different panel locations began to diverge. It

was demonstrated through correlations of optical density absorbance wavelengths (OD), specifically OD<sub>660</sub>, OD<sub>680</sub>, and OD<sub>735</sub>, as well as weekly dry weight correlations, that changes in OD were indeed because of changes in the amount of biomass, and not the change in chlorophyll-a inside each cell (paper II). It is possible that these diverging optical densities represent cyclical effects of anisotropic light or temperature gradients, where the densely-packed panels of the PBR module may thermally insulate the microalgae in the colder hours of the night in the inner panels of the reactor module.

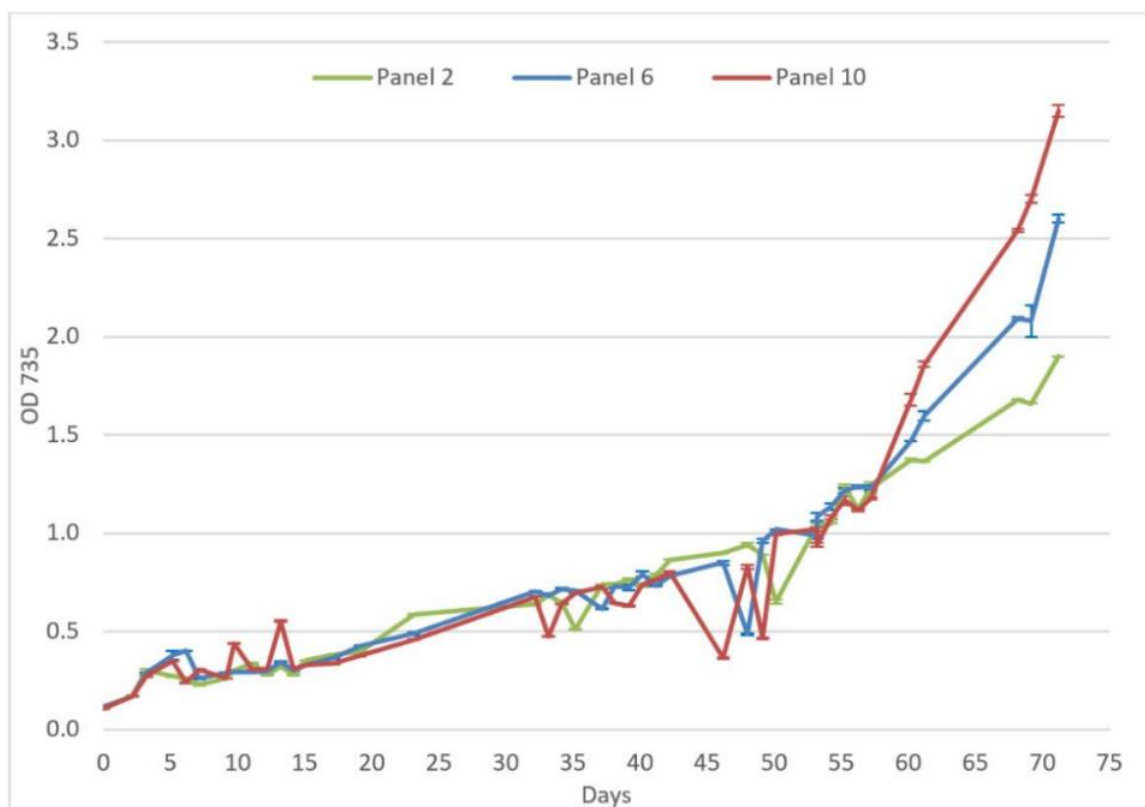


Figure 12. The divergence of biomass (optical density 735 nm) at three different panel sampling locations.

In the effort to address the possible technologies both capable of measuring a variety of molecules, being multiplexed to monitor several reactor locations to monitor inhomogeneities, and capable of real-time monitoring to improve process automation, paper III addresses the promising intersection of three disciplines: microalgae biorefinery symbiosis (MBS), vibrational spectroscopy, and machine learning. Of the numerous technologies with on-line/in-line potential, mid-infrared (MIR), near-infrared (NIR) absorbance spectroscopy, and Raman scattering spectroscopy showed the most potential for monitoring the above criteria. Altogether, these spectroscopies are referred

to as “vibrational spectroscopy” referring to the vibration and rotations of molecular bonds in response to electromagnetic stimulation. These spectroscopies can be used to measure numerous compounds in microalgae including biomass, lipids, proteins, carbohydrates, chlorophyll, and carotenoids as well as other HVPs (Table 2 & Table 3). With advances in chemometric computing tools, vibrational spectroscopies can also be used to qualitatively measure contamination of competing microalgal species or other microbes and differentiate microalgal strains at a phenotypic level from spectral aberrations, as well. Overall, collection of real-time on-line/in-line spectral data can improve multivariate process control (MVPC) or model predictive control (MPC), whereby the real-time vibrational spectra data of molecular compounds are correlated—linearly or non-linearly—to lab-determined values of those compounds, which can then be used to model and automate the highly dynamic system. In the above scenario, robust MVPC models can then be correlated to permanent the interplay of common microalgae production CPPs (pH, temperature, irradiance). In doing so, productions can conceivably rely on a virtual model and the on-line/in-line probe can be removed. These so-called “software sensors” or “soft-sensors” are merely virtual molecular sensors, without hardware, based in a computer that are correlated to measured parameters that can reveal the state of the system.

In general, the objective of the review paper III was to establish the broad initial assessment of high-selectivity vibrational spectroscopy for its use in on-line/in-line, real-time monitoring of an outdoor MBS and the general contribution of vibrational spectroscopy to microalgae biotechnology, as a whole. (Table 2 & Table 3). The same technologies have been used to infer important microalgal critical process parameters (CPPs) such pH and photosynthetic efficiency (Meng et al., 2014; Schenk et al., 2008), with the possibility of replacing these CPPs instead of augmenting them. Whether on-line, in-line, at-line, or off-line, vibrational spectroscopy has a promising outlook for improving microalgae biotechnology academically and industrially to better understand the entire MBS process for modeling to improve product quality. The critical review in paper III includes a comprehensive list of *in vivo* applications of these vibrational spectroscopies to promote and incentivize on-line/in-line research (Table 2).

Of the numerous *in vivo* studies summarized in paper III, there are several noteworthy technological advances in microalgae PAT that are also mirrored in other similar, cellular suspension bioprocesses to a greater degree. Cur-

rently, there is extensive literature of off-line vibrational spectroscopy of microalgae monitoring carbohydrates, proteins, lipids, and pigments both quantitatively and qualitatively (Table 2 & Table 3). Most of this literature is focused on understanding physiological changes in microalgae during environmental stresses as well as differentiating between microalgae at a phenotypic, strain, and species level, which may advance towards monitoring contaminating of competing microbial and microalgal species. Though offline samples are highly controlled and often denser than microalgal suspensions, on-line monitoring using vibrational spectroscopy is starting to burgeon in microalgae applications, while persevering in other bioprocess industries. Meanwhile, spectral databases from off-line studies continue to grow, which can subsequently benefit on-line vibrational spectroscopy chemometrics. ATR-FTIR, a variation of MIR, has been used to monitor intercellular sugars (lactose, glucose, and galactose) in WW media sustaining the microalgae *Scenedesmus obliquus*, *in situ* with a fiber optic probe (Girard et al., 2013) and more recent attempts have been made to create online ATR-FTIR for monitoring *Dunaliella parva* (Vogt and White, 2015). ATR-FTIR spectra have also been used for *in situ* monitoring of other bioindustries to measure optical density and a number of other intracellular compounds such as ammonium, phosphates, acetate, glucose, fructose, organic acids and a multitude of other bioproducts. On the other hand, a large majority of *in vivo* microalgae vibrational spectroscopy studies are performed with Raman spectroscopy. To date, Raman spectroscopy has been used to measure glucose and biomass on-line in heterotrophically grown *Auxenochlorella prototheocoides* (Nadadoor et al., 2012), and extracellular polymeric substances in *Porphyridium purpureum* (Noack et al., 2013). In other bioindustries, Raman has been used to measure intracellular carotenoids and intercellular glucose, acetate, ethanol, nitrate, and nitrite (Lourenço et al., 2012). Unlike MIR and NIR, which have a precedent in on-line bioprocess analysis, the advent of tunable/notch filters, tunable lasers, and improved computing for chemometric analysis has shifted attention towards Raman spectroscopy as a potential on-line/in-line monitoring tool. Dispersive vibrational spectroscopy such as most applications of NIR and Raman have high potential to be modularized and simplified with improving technology and computational power due to their absence of interferometers in Fourier transform (FT) instruments, such as those used in ATR-FTIR. The most widespread use of on-line vibrational spectroscopy is NIR spectroscopy. On-line NIR has been used for the measurement of protein, extracellular protein, pyruvate, viscosity, acetic acid, total sugars, oil, phosphate, astaxanthin, glycerol, nitrogen sources, and casein (De Beer et al., 2011; He and Sun, 2015; Huang et al., 2008; Landgrebe et al., 2010; Lourenço et al., 2012; Porep et al., 2015).

Currently, real-time on-line/in-line monitoring is most frequently used in an academic setting and is looked as difficult when monitoring large-scale systems when considering the maintenance, calibration, and life-span of monitoring tools when exposed to the elements. However, recently, hardware and software modeling improvements have brought vibrational spectroscopy closer to reality at large-scales. Acquiring real-time data of several molecules critical quality attributes (CQAs) simultaneously through vibrational spectroscopy and correlating these data to critical process parameters (CPPs) such as pH, temperature, and local irradiance in an outdoor bioreaction can be used to circumvent the need for permanent, indirect, real-time CQA monitoring. Theoretically, using temporally segmented modeling, these instruments can be multiplexed with multiple fiber optic conduits or resituated at several reactor locations and then removed once a robust model of the system has been built with a large, representative data set. Recently, software sensors, or soft-sensors, have been studied to estimate real-time lipids from on-line microalgal biomass measurements and infrequent off-line glucose and lipid analysis (Yoo et al., 2015). Soft-sensors have also been used to predict real-time ammonium, and nitrate + nitrite concentrations in WW activated sludge systems (Cecil and Kozłowska, 2010). These software sensor studies generally rely on correlating infrequent off-line sampling with on-line CPPs; however, correlating real-time spectra of a variety of molecules from vibrational spectroscopy may significantly improve soft-sensor modeling of multiple analytes, especially in the complex MBS. Following model development and implementing the soft-sensor, vibrational spectroscopic probes can be removed, mitigating concerns of long-term probe maintenance (Cecil and Kozłowska, 2010) and contamination.



Table 2. A comprehensive overview of wet/*in vivo* vibrational spectroscopy wet/*in vivo* applications in microalgal research. Numbered references are contained within the cell for the respective works and are referenced in the bibliography of paper III.

Microalgal species	Mode	Reference																	Compositional analysis	Growth and Nutrient dynamics	Strain selection/screening	Classification/differentiation	Bio-prospecting	Ecotoxicological/metal complexing	Biomass/productivity	Protein	Carbohydrates/ Starch	Lipids	Lipid unsaturation	Cellulose	Pigments	Chlorophylls	Carotenoids	Astaxanthin	Sulfated EPS
<i>Auxenochlorella protothecoides</i> (UTEX B25) [hetero]	Raman	(Nadadoor et al., 2012)																																	
<i>Botryococcus braunii</i>	Micro-Raman	(Weiss et al., 2010)																																	
<i>Botryococcus sudeticus</i> Lemmermann (CCALA 780)	Micro-Raman	(Samek et al., 2010)																																	
<i>Brachiomonas submarina</i> (BRACH)	Raman	(Brahma et al., 1983)																																	
<i>Calothrix</i> sp.	FTIR	(Yee et al., 2004)																																	
<i>Chaetoceros muelleri</i>	Portable Raman	(Wood et al., 2005)																																	
<i>Chaetoceros sociale</i> f. <i>radians</i> (P10H)	Raman	(Brahma et al., 1983)																																	
<i>Chaetoceros tercs</i> (M4L)	Raman	(Brahma et al., 1983)																																	
<i>Chlamydomonas reinhardtii</i>	Micro-Raman	(Wu et al., 2011)																																	
<i>Chlamydomonas reinhardtii</i> (CC124)	Micro-RACS	(Ji et al., 2014)																																	
<i>Chlamydomonas reinhardtii</i> (CC125 strain mt+)	Micro-Raman	(Kubo et al., 2000)																																	
<i>Chlamydomonas reinhardtii</i> (CC4324)	Micro-RACS	(Ji et al., 2014)																																	
<i>Chlamydomonas reinhardtii</i> (CC4333)	Micro-RACS	(Ji et al., 2014)																																	
<i>Chlamydomonas</i> sp. (CCALA)	Micro-Raman	(Samek et al., 2010)																																	
<i>Chlamydomonas</i> sp. D	Raman	(Wu et al., 1998)																																	
<i>Chlorella capsulata</i> (FLAE)	Raman	(Wu et al., 1998)																																	
<i>Chlorella pyrenoidosa</i> (FACHB-9)	micro-RACS	(Ji et al., 2014)																																	
<i>Chlorella vulgaris</i>	Vis-SW-NIR	(Challagulla et al., 2014)																																	
<i>Chlorella vulgaris</i> (ESP-31)	Raman	(Lee et al., 2013)																																	
<i>Chlorella vulgaris</i> Beijernick	ATR-FTIR	(Vogel et al., 2010)																																	
<i>Ckactoceros soeiale</i> f. <i>radians</i> (B5)	Raman	(Brahma et al., 1983)																																	
<i>Ckactoceros soeiale</i> f. <i>radians</i> (B7)	Raman	(Brahma et al., 1983)																																	
<i>Coccomyxa subellipsoidea</i> (C169)	CARS	(He et al., 2012)																																	
<i>Cricosphaera roscoffensis</i> (LB940)	Raman	(Wu et al., 1998)																																	
<i>Dunaliella salina</i>	Micro-Raman LT	(Davis et al., 2014)																																	
<i>Dunaliella parva</i> (CCAP 19/9)	HATR-FTIR	(Vogt and White, 2015)																																	
<i>Dunaliella salina</i> (LB200)	Raman	(Wu et al., 1998)																																	
<i>Dunaliella tertiolecta</i>	Raman	(Heraud et al., 2007, 2006)																																	
	Portable Raman	(Wood et al., 2005)																																	
<i>Dunaliella tertiolecta</i> (DUN)	Raman	(Wu et al., 1998)																																	
	Raman	(Brahma et al., 1983)																																	
	Micro-Raman	(Pilát et al., 2012, 2011)																																	



Table 3. A comprehensive overview of off-line vibrational spectroscopy applications in microalgal research. Numbered references are contained within the cell for the respective works and are referenced in the bibliography of paper III.

Microalgal species	Mode	Reference	Compositional analysis	Growth/ N + P uptake	Strain selection/screening	Classification/differentiation	Bio-prospecting	Ecotoxicological/metal complexing	Biomass/productivity	Protein	Carbohydrates	Lipids	Fatty acids	Cellulose	Pigments	Chlorophyll	Carotenoids	Astaxanthin	EPS	Silica	Spectral aberration	ASH	Nitrogen	Phosphorus	Gross Calorific Value	TKN	Nucleic Acid	Phospholipid	Phosphorylated compounds
<i>Acaryochloris marina</i>	Raman	(Chen et al., 2004)																											
Algal Turf Scrubber Sample	FTIR	(Mulbry et al., 2012)																											
	FT-NIR	(Mulbry et al., 2012)																											
<i>Anabaena variabilis</i> Mon	FTIR	(Kansiz et al., 1999)																											
<i>Botryococcus braunii</i> (FACHB-357)	FTIR	(Feng et al., 2013)																											
<i>Chaetoceros muelleri</i>	FTIR	(Liang et al., 2006)																											
	FTIR	(Giordano, 2001)																											
<i>Chaetoceros</i> sp.	FTIR	(Meng et al., 2014)																											
<i>Chlamydomonas reinhardtii</i>	Micro-FTIR	(Dean et al., 2008)																											
	Micro-FTIR	(Dean et al., 2010)																											
<i>Chlamydomonas reinhardtii</i> (CC124)	SERS	(Ayas et al., 2013)																											
<i>Chlamydomonas reinhardtii</i> (CC125)	FTIR	(Giordano et al., 2009)																											
<i>Chlamydomonas reinhardtii</i> (SAG 1132b)	FTIR	(Wagner et al., 2010)																											
<i>Chlorella emersonii</i>	FTIR	(Kansiz et al., 1999)																											
<i>Chlorella marina</i> (CCAP 211/27)	FTIR	(Giordano et al., 2009)																											
<i>Chlorella protothecoides</i> (CSIROCS-41)	NIR	(Liu et al., 2015)																											
<i>Chlorella pyrenoidosa</i>	FTIR	(Feng et al., 2013)																											
	FTIR	(Feng et al., 2013)																											
	FTIR	(Meng et al., 2014)																											
<i>Chlorella sorokiniana</i> (UTEX #1230)	Micro-Raman	(Huang et al., 2010)																											
<i>Chlorella</i> sp. 1	FTIR	(Odlare et al., 2011)																											
<i>Chlorella</i> sp. 2	NIR	(Laurens and Wolfrum, 2013)																											
<i>Chlorella viridis</i>	FTIR	(Pistorius et al., 2009)																											
<i>Chlorella vulgaris</i>	FTIR	(Feng et al., 2013)																											
	Vis-SW-NIR	(Challagulla et al., 2014)																											
	FT-NIR	(Challagulla et al., 2014)																											
<i>Chlorella vulgaris</i> (Carolina 15-2075) [heterotrophic]	NIR	(Liu et al., 2015)																											
<i>Chlorella vulgaris</i> bey (FACHB-1227)	FTIR	(Feng et al., 2013)																											
<i>Chlorella zofingiensis</i> (ATCC 30412) [heterotrophic]	NIR	(Liu et al., 2015)																											
<i>Chlorococcum</i> sp.	NIR	(Laurens and Wolfrum, 2011)																											
	ATR-FTIR	(Laurens and Wolfrum, 2011)																											

Microalgal species	Mode	Reference																		
			Compositional analysis	Growth/ N + P uptake	Strain selection/screening	Classification/differentiation	Bioprospecting	Ecotoxicological/metal complexing	Biomass/productivity	Protein	Carbohydrates	Lipids	Fatty acids	Cellulose	Pigments	Chlorophyll	Carotenoids	EPS	Silica	Spectral alteration
<i>Croococcus minutus</i>	Micro-FTIR	(Stehfest et al., 2005)																		
<i>Cyanidium caldarium</i>	FTIR	(Pistorius et al., 2009)																		
<i>Cyclotella meneghiniana</i>	Micro-FTIR	(Stehfest et al., 2005)																		
<i>Dunaliella parva</i> (CCAP 19/9)	FTIR	(Giordano et al., 2009)																		
<i>Dunaliella salina</i>	FTIR	(Liu et al., 2013)																		
<i>Dunaliella salina</i> (CCAP 19/25)	FTIR	(Giordano et al., 2009)																		
<i>Dunaliella tertiolecta</i>	ATR-FTIR	(Mecozzi et al., 2007)																		
	ATR-FTIR	(Jiang et al., 2012)																		
<i>Emiliania huxleyi</i> (CCAP 920/11)	FTIR	(Giordano et al., 2009)																		
<i>Euglena gracilis</i> UTEX 368	Micro-FTIR	(Hirschmugl et al., 2006)																		
<i>Galdiera sulfuraria</i>	FTIR	(Pistorius et al., 2009)																		
<i>Haematococcus pluvialis</i> FACHB-712 (mutants)	FTIR	(Liu and Huang, 2016)																		
	Micro-Raman	(Liu and Huang, 2016)																		
<i>Isochrysis galbana</i> (CCMP 1323)	FTIR	(Giordano et al., 2009)																		
<i>Isochrysis</i> sp.	FTIR	(Meng et al., 2014)																		
<i>Isochrysis zhangjiangensis</i>	FTIR	(Meng et al., 2014)																		
<i>Kirchneriella</i> sp.	FT-NIR	(Brown et al., 2014)																		
<i>Microcystis aeruginosa</i>	Micro-FTIR	(Stehfest et al., 2005)																		
<i>Microcystis aeruginosa</i> (DTW)	FTIR	(Kansiz et al., 1999)																		
<i>Microcystis aeruginosa</i> (FACHB-940)	FTIR	(Feng et al., 2013)																		
<i>Microcystis aeruginosa</i> (LB-A1)	FTIR	(Kansiz et al., 1999)																		
<i>Nannochloris</i> sp.	NIR	(Ge, 2016)																		
	ATR-FTIR	(Ge, 2016)																		
<i>Nannochloropsis oculata</i>	FTIR	(Coat et al., 2014)																		
<i>Nannochloropsis</i> sp.	NIR	(Ge, 2016)																		
	ATR-FTIR	(Ge, 2016)																		
<i>Nannochloropsis</i> sp. (CCAP 211/78)	ATR-FTIR	(Mayers et al., 2013)																		
<i>Nannochloropsis</i> sp. 1	NIR	(Laurens and Wolfrum, 2013)																		
<i>Nannochloropsis</i> sp. 2	Micro-FTIR	(Tan et al., 2013)																		
<i>Nannochloropsis</i> sp. 3	FTIR	(Feng et al., 2013)																		
<i>Nannochloropsis</i> sp. 4	FT-NIR	(Brown et al., 2014)																		
<i>Nannochloropsis</i> sp. 5	NIR	(Laurens and Wolfrum, 2011)																		
<i>Nannochloropsis</i> sp. 5	ATR-FTIR	(Laurens and Wolfrum, 2011)																		

Microalgal species	Mode	Reference																										
			Compositional analysis	Growth/ N + P uptake	Strain selection/screening	Classification/differentiation	Bio-prospecting	Ecotoxicological/metal complexing	Biomass/productivity	Protein	Carbohydrates	Lipids	Fatty acids	Cellulose	Pigments	Chlorophyll	Carotenoids	Astaxanthin	EPS	Silica	Spectral aberration	ASH	Nitrogen	Phosphorus	Gross Calorific Value	Nucleic Acid	Phospholipid	Phosphorylated compounds
Navicula sp.1	Vis-SW-NIR	(Challagulla et al., 2014)																										
Navicula sp.1	FT-NIR	(Challagulla et al., 2014)																										
Navicula sp.2	Vis-SW-NIR	(Challagulla et al., 2014)																										
Navicula sp.2	FT-NIR	(Challagulla et al., 2014)																										
Neochloris oleoabundans (UTEX 1185)	Micro-Raman	(Huang et al., 2010)																										
Nitzschia sp.	Micro-FTIR	(Beardall et al., 2001)																										
Nitzschia pusilla	Vis-SW-NIR	(Challagulla et al., 2014)																										
	FT-NIR	(Challagulla et al., 2014)																										
Nostoc sp.	Micro-FTIR	(Stehfest et al., 2005)																										
Oscillatoria sp.	NIR	(Kansiz et al., 1999)																										
Ostreopsis ovata (CBA-T)	FTIR	(Mecozzi et al., 2011)																										
Ostreopsis ovata (CBA-T)	FT-NIR	(Mecozzi et al., 2011)																										
Pediastrum duplex Meyen	FTIR	(Sigee et al., 2002)																										
Phaeodactylum tricornutum	FTIR	(Pistorius et al., 2009)																										
	Micro-FTIR	(Stehfest et al., 2005)																										
	FTIR	(Liang et al., 2006)																										
Phaeodactylum tricornutum (CCAP 1052/1A)	FTIR	(Giordano et al., 2009)																										
Phormidium autumnale (CCAP 1462/10)	FTIR	(Giordano et al., 2009)																										
Phormidium luridum var olivace Boresch	Micro-FTIR	(Beardall et al., 2001)																										
Porphyridium cruentum	FTIR	(Meng et al., 2014)																										
	FTIR	(Meng et al., 2014)																										
Porphyridium purpureum	ATR-FTIR	(Fuentes-Grünwald et al., 2015)																										
Protoceratium reticulatum (PRA 0206)	FTIR	(Giordano et al., 2009)																										
Rhopalosolen saccatus	FT-NIR	(Challagulla et al., 2016b)																										
Scenedesmus obliquus	FTIR	(Pistorius et al., 2009)																										
	FTIR	(Meng et al., 2014)																										
Scenedesmus quadricauda	Micro-FTIR	(Beardall et al., 2001)																										
Scenedesmus quadricauda (Turpin) Breb	Micro-FTIR	(Heraud et al., 2008)																										
Scenedesmus sp.	NIR	(Laurens and Wolfrum, 2013)																										
Scenedesmus subspicatus	Micro-FTIR	(Dean et al., 2010)																										
	FTIR	(Sigee et al., 2007)																										
Skeletonema marinoi (CCMP 2092)	FTIR	(Giordano et al., 2009)																										
Sphaerocystis schroeteri Choda	Micro-FTIR	(Beardall et al., 2001)																										
Spirulina platensis	FTIR	(Pistorius et al., 2009)																										
Spirulina sp.	NIR	(Laurens and Wolfrum, 2011)																										
	ATR-FTIR	(Laurens and Wolfrum, 2011)																										
Symbiodinium microadriaticum	FTIR	(Giordano et al., 2009)																										
Synechococcus sp.	FTIR	(Kansiz et al., 1999)																										
Synechococcus sp. (PCC7942)	FTIR	(Giordano et al., 2009)																										
Synechococcus sp. (PCC7942)	HATR-FTIR	(Silva et al., 2014)																										
Tetraselmis suecica (PCC 305)	FTIR	(Giordano et al., 2009)																										
Thalassiosira pseudonana	ATR-FTIR	(Jiang et al., 2012)																										
Thalassiosira weissflogii (PCC 9115)	FTIR	(Giordano et al., 2009)																										

Laser-induced breakdown spectroscopy (LIBS) demonstrated in the field of microalgae biotechnology (Pořízka et al., 2012) has also emerged as a high-selectivity technology for chemical detection in submersible settings (Galbács, 2015; Goueguel et al., 2014; Hahn and Omenetto, 2012; Haider et al., 2014; Latkoczy et al., 2010; Lazic and Jovičević, 2014; Pořízka et al., 2014; Yu et al., 2014); however, it is in the very early stages of development. Using liquid jets and nanosecond laser pulse LIBS, Pořízka et al. (2012) successfully discriminated four microalgal strains (*Chlamydomonas reinhardtii*, *Chlorococuum zurek*, *Desmodesmus quadricus*, *Haematococcus pluvialis*) from each other based on LIBS spectral data of Mg, Ca, Na, and K. Like vibrational spectroscopy, LIBS may elucidate rich biological data of the microalgae under cultivation but also contamination from WW media. Currently, the use of LIBS in microalgal biotechnology remains largely unexplored, however *in situ*, on-line, real-time use of LIBS is promising (review: Pořízka et al., 2014); however, the extent of research pales in comparison NIR, MIR, and Raman spectroscopy research in biotechnology.

Apart from MIR, NIR, and Raman spectroscopies, there are some advanced CQA tools that can be used to monitor microalgae on-line/in-line; however, some of these technologies may not be economical or scalable in an MBS with their relatively low-selectivity compared to vibrational spectroscopy. In microalgae productions today, fluorescence spectroscopy is widely used as a tool to monitor the photosynthetic efficiency (PE) of microalgae and is done so through induction/relaxation kinetics or pulse amplitude modulation (PAM), the latter of which can be used on-line because it does not require dark adaptation (Huot and Babin, 2010; Malapascua et al., 2014). However, these methods do not account for respiration (Malapascua et al., 2014) integral to the understanding of mixotrophy, heterotrophy, and respiration at night in outdoor reactions. Furthermore, inferences such as nutrient availability and nutrient-induced fluorescent transitions (NIFTS) and their relationship to photosynthetic efficiency (PE) in microalgae can be influenced by the prior light and UV exposure (Beardall et al., 2001), which are changing and inconsistent in outdoor reactions. Similarly, nutrient uptake protocols inferring lipid synthesis/accumulation from fluorescence can be affected by process parameters such as incident light, temperature, and carbon availability (Coat et al., 2014). Very thorough lipid measurements can be made through technology such as nuclear magnetic resonance (NMR) spectroscopy (Beal et al., 2010; Chauton et al., 2003; Danielewicz et al., 2011; Davey et al., 2012; Gao et al., 2008; Nuzzo et al., 2013; Skogen Chauton et al., 2003); however,

it is expensive and impractical for a single analyte. Dielectric spectroscopy (Bono et al., 2013; Justice et al., 2011) can elucidate information about microalgal dry weight, health, viability, and non-viability, and infer lipid concentrations (Bono et al., 2013), to some degree, but it cannot be used to measure or infer other molecules. In the end, these specified instruments do not demonstrate the potential to simultaneously characterize several industrially important microalgae macromolecules quantitatively or qualitatively. To that end, these specific technologies do not demonstrate the ability to transcend their low-selectivity to identify more unknowns, which is inherent to vibrational spectroscopy of molecular bonds.

Overall, microalgal productions remain complicated, and the need of time-resolute macromolecule monitoring can be of the utmost importance to understand microalgae metabolism for optimizing microalgae bioreactions (Wagner et al., 2010). It follows that standard CQA monitoring tools may not be able to compete with high-selectivity of vibrational spectroscopy, which has the potential to directly or indirectly monitor most, if not all, of the standard CQAs monitored in microalgae productions in the future. Moreover, with the development of robust machine learning MVPC trained on time-resolute vibrational spectroscopy data, all of the above technologies may even be replaced by software sensors (paper III). With hope, the recognition of vibrational spectroscopy as a viable monitoring tool to meet PAT guidelines in several bioindustries will be realized in microalgae productions. Complex and dynamic outdoor PBRs, alone, are enough to necessitate real-time monitoring of various parameters to improve microalgae production, and the need to monitor microalgae in a PBR to meet quality and regulatory needs to ultimately valorize the MBS make the need even more pressing.

## 7 Conclusions

This thesis has outlined several novel ways to optimize and valorize large-scale microalgal productions in an MBS concept from screening, to production, to production optimization, from end-to-end. The results from research summarized in this thesis demonstrate the screening of viable microalgae for growth different nitrogen sources, to testing these microalgae at large-scales on wastewater (WW), to recognizing the academic limitations of large-scale studies and how these concerns can be addressed with novel technologies. The main conclusions of this thesis work are as follows:

- In the screening of variable microalgal species for an MBS, low detection of microalgal chlorophyll-a autofluorescence *in vivo* in 24-well microplates can be used to detect specific growth rates (SGRs) of microalgae before large physiochemical changes from nutrient assimilation occur in several microalgal species (paper I).
- The chlorophyll-a autofluorescence method is capable of high-throughput detection of SGRs and acclimatization of microalgae to various nitrogen sources and identifying robust microalgae capable of rapid acclimatization to fluctuating nitrogen sources (paper I).
- Novozymes bioindustrial WW was identified as a viable bioindustrial WW source for microalgal productions in an MBS. The WW maintained nutrient content after removal of biological contaminants through ultrafiltration and sustained microalgal growth. Despite the need for nutrient supplementation during the experiment, the WW was not demonstrably deleterious to microalgae growth due to other chemical species (paper II).
- The robust screened microalgae *Chlorella sorokiniana* can be grown on bioindustrial WWs containing different nitrogen sources at low temperature and irradiance; however, low temperature and irradiance may severely inhibit the adaptation of the microalgae *C. sorokiniana* from ammonium to nitrate metabolism (paper I & II).
- By comparing microalgal productions on defined freshwater media (autotrophic) and WW media containing organic carbon (mixotrophic) in similar temperature and irradiance, it was shown that mixotrophic growth might benefit microalgal productions in low light and temperature environments compared to pure autotrophic growth.



- Microalgae could be harvested/up-concentrated through microfiltration; however, there was a loss in cell viability from prolonged exposure of microalgal cells to the membrane surface. The results demonstrate that the loss of viability may also come with a loss of valuable bioproducts in an MBS.
- On-line/in-line mid-infrared, near-infrared, and Raman vibrational spectroscopy process analytical technology (PAT) are possible tools for monitoring the gamut of microalgae macromolecules of industrial interest in an MBS. These tools have the possibility to improve fractionation of compounds, monitor contamination, and promote model development for highly dynamic photobioreactor systems with spatial, temporal, chemical, and metabolic inhomogeneities caused by anisotropic reactor designs, fluctuating light, temperatures, and WW composition. Ultimately these tools can improve and may be necessary to valorize the complex MBS.
- Vibrational spectroscopy is a promising optical, on-line, monitoring technology that can also be used to infer important microalgal critical process parameters (CPPs) such pH and photosynthetic efficiency, with the possibility of replacing these CPPs instead of augmenting them.
- Big data from on-line/in-line monitoring of macromolecules can be used to improve process automation through multivariate process control (MVPC). The evolution of MVPC may lead to robust software sensors capable of replacing on-line/in-line CQA monitoring, altogether.

## 8 References

- Abu-Ghosh, S., Fixler, D., Dubinsky, Z., Iluz, D., 2015. Flashing light in microalgae biotechnology. *Bioresour. Technol.* 203, 357–363. doi:10.1016/j.biortech.2015.12.057
- Ahluwalia, S.S., Goyal, D., 2007. Microbial and plant derived biomass for removal of heavy metals from wastewater. *Bioresour. Technol.* 98, 2243–2257. doi:10.1016/j.biortech.2005.12.006
- Arranz, A., Bordel, S., Villaverde, S., Zamarreño, J.M., Guieysse, B., Muñoz, R., 2008. Modeling photosynthetically oxygenated biodegradation processes using artificial neural networks. *J. Hazard. Mater.* 155, 51–57. doi:10.1016/j.jhazmat.2007.11.027
- Babel, S., Takizawa, S., 2010. Microfiltration membrane fouling and cake behavior during algal filtration. *Desalination* 261, 46–51. doi:10.1016/j.desal.2010.05.038
- Bates, S.S., 1976. Effects of light and ammonium on nitrate uptake by two species of estuarine phytoplankton. *Limnol. Oceanogr.* 21, 212–218. doi:10.2307/2835221
- Baughman, D.R., Liu, Y.A. (Yih A., 1995. *Neural networks in bioprocessing and chemical engineering*. Academic Press.
- Beal, C.M., Webber, M.E., Ruoff, R.S., Hebner, R.E., 2010. Lipid analysis of *Neochloris oleoabundans* by liquid state NMR. *Biotechnol. Bioeng.* 106, 573–583. doi:10.1002/bit.22701
- Beardall, J., Berman, T., Heraud, P., Omo Kadiri, M., Light, B.R., Patterson, G., Roberts, S., Sulzberger, B., Sahan, E., Uehlinger, U., Wood, B., 2001. A comparison of methods for detection of phosphate limitation in microalgae. *Aquat. Sci.* 63, 107–121. doi:10.1007/PL00001342
- Becker, E.W., 2013. Microalgae for Aquaculture: Nutritional Aspects. *Handb. Microalgal Cult. Appl. Phycol. Biotechnol.* 671–691. doi:10.1002/9781118567166.ch36
- Becker, E.W., 1994. *Cambridge Studies in Biotechnology, Vol. 10. Microalgae: Biotechnology and Microbiology*. Cambridge University Press.
- Becker, W.E., 2013. Microalgae in Human and Animal Nutrition. *Handb. Microalgal Cult. Appl. Phycol. Biotechnol.* 461–503. doi:10.1002/9780470995280.ch18

- Bernard, O., Mairet, F., Chachuat, B., 2015. Modelling of Microalgae Culture Systems with Applications to Control and Optimization, in: *Advances in Biochemical Engineering/biotechnology*. pp. 59–87. doi:10.1007/10\_2014\_287
- Bohutskyi, P., Bouwer, E., 2013. Biogas Production from Algae and Cyanobacteria Through Anaerobic Digestion. A Review, Analysis, and Research Needs, *Advance Biofuels and Bioproducts*. Vol 1. doi:10.1007/978-1-4614-3348-4
- Bono, M.S., Ahner, B.A., Kirby, B.J., 2013. Detection of algal lipid accumulation due to nitrogen limitation via dielectric spectroscopy of *Chlamydomonas reinhardtii* suspensions in a coaxial transmission line sample cell. *Bioresour. Technol.* 143, 623–631. doi:10.1016/j.biortech.2013.06.040
- Borowitzka, M. a., 2013. High-value products from microalgae—their development and commercialisation. *J. Appl. Phycol.* 25, 743–756. doi:10.1007/s10811-013-9983-9
- Brune, D.E., Lundquist, T., Benemann, J., n.d. *Algal Production and Harvest for Food , Feed and Biofuels Objectives ; High Rate Photosynthetic Systems Increasing Energy Independence Water requirements GHG reduction through carbon - neutral food ,*. Carbon N. Y.
- Camacho, F.G., Gomez, A.C., Sobczuk, T.M., Grima, E.M., 2000. Effects of mechanical and hydrodynamic stress in agitated, sparged cultures of *Porphyridium cruentum*. *Process Biochem.* 35, 1045–1050. doi:10.1016/S0032-9592(00)00138-2
- Campbell, A., Doswald, N., 2009. The impacts of biofuel production on biodiversity: A review of the current literature.
- Campbell, P.K., Beer, T., Batten, D., 2011. Life cycle assessment of biodiesel production from microalgae in ponds. *Bioresour. Technol.* 102, 50–56. doi:10.1016/j.biortech.2010.06.048
- Carmichael, W.W., 1994. The Toxins of Cyanobacteria. *Sci. Am.* 270, 78–86. doi:10.1038/scientificamerican0194-78
- Cecil, D., Kozłowska, M., 2010. Software sensors are a real alternative to true sensors. *Environ. Model. Softw.* 25, 622–625. doi:10.1016/j.envsoft.2009.05.004
- Celikovsky, S., Papacek, S., Cervantes-Herrera, A., Ruiz-Leon, J., 2010. Singular Perturbation Based Solution to Optimal Microalgal Growth Problem and Its Infinite Time Horizon Analysis. *IEEE Trans. Automat. Contr.* 55, 767–772. doi:10.1109/TAC.2010.2040498
- Chadwick, D.T., McDonnell, K.P., Brennan, L.P., Fagan, C.C., Everard, C.D.,

2014. Evaluation of infrared techniques for the assessment of biomass and biofuel quality parameters and conversion technology processes: A review. *Renew. Sustain. Energy Rev.* 30, 672–681. doi:10.1016/j.rser.2013.11.006
- Chauton, M.S., St\orseth, T.R., Johnsen, G., 2003. High-resolution magic angle spinning  $^1\text{H}$  NMR analysis of whole cells of *Thalassiosira pseudonana* (Bacillariophyceae): Broad range analysis of metabolic composition and nutritional value. *J. Appl. Phycol.* 15, 533–542. doi:10.3354/meps256057
- Chini Zittelli, G., Rodolfi, L., Biondi, N., Tredici, M.R., 2006. Productivity and photosynthetic efficiency of outdoor cultures of *Tetraselmis suecica* in annular columns. *Aquaculture* 261, 932–943. doi:10.1016/j.aquaculture.2006.08.011
- Coat, R., Montalescot, V., León, E.S., Kucma, D., Perrier, C., Jubeau, S., Thouand, G., Legrand, J., Pruvost, J., Gonçalves, O., 2014. Unravelling the matrix effect of fresh sampled cells for in vivo unbiased FTIR determination of the absolute concentration of total lipid content of microalgae. *Bioprocess Biosyst. Eng.* 37, 2175–2187. doi:10.1007/s00449-014-1194-5
- Cogne, G., Gros, J.-B., Dussap, C.-G., 2003. Identification of a metabolic network structure representative of *Arthrospira (Spirulina) platensis* metabolism. *Biotechnol. Bioeng.* 84, 667–676. doi:10.1002/bit.10808
- Coles, J.F., Jones, R.C., 2000. Effect of temperature on photosynthesis-light response and growth of four phytoplankton species isolated from a tidal freshwater river. *J. Phycol.* 36, 7–16. doi:10.1046/j.1529-8817.2000.98219.x
- Collet, P., Hélias, A., Lardon, L., Ras, M., Goy, R.-A., Steyer, J.-P., 2011. Life-cycle assessment of microalgae culture coupled to biogas production. *Bioresour. Technol.* 102, 207–214. doi:10.1016/j.biortech.2010.06.154
- Cordell, D., Drangert, J.-O., White, S., 2009. The story of phosphorus: Global food security and food for thought. *Glob. Environ. Chang.* 19, 292–305. doi:10.1016/j.gloenvcha.2008.10.009
- Craggs, R.J., Heubeck, S., Lundquist, T.J., Benemann, J.R., 2011. Algal biofuels from wastewater treatment high rate algal ponds. *Water Sci. Technol.* 63.
- Danielewicz, M. a., Anderson, L. a., Franz, a. K., 2011. Triacylglycerol profiling of marine microalgae by mass spectrometry. *J. Lipid Res.* 52, 2101–2108. doi:10.1194/jlr.D018408
- Davey, P.T., Hiscox, W.C., Luckner, B.F., O’Fallon, J. V., Chen, S., Helms,

- G.L., 2012. Rapid triacylglyceride detection and quantification in live micro-algal cultures via liquid state  $^1\text{H}$  NMR. *Algal Res.* 1, 166–175. doi:10.1016/j.algal.2012.07.003
- de-Bashan, L.E., Bashan, Y., 2010. Immobilized microalgae for removing pollutants: Review of practical aspects. *Bioresour. Technol.* 101, 1611–1627. doi:10.1016/j.biortech.2009.09.043
- De Beer, T., Burggraef, A., Fonteyne, M., Saerens, L., Remon, J.P., Vervaet, C., 2011. Near infrared and Raman spectroscopy for the in-process monitoring of pharmaceutical production processes. *Int. J. Pharm.* 417, 32–47. doi:10.1016/j.ijpharm.2010.12.012
- Degen, J., Uebele, A., Retze, A., Schmid-Staiger, U., Trösch, W., 2001. A novel airlift photobioreactor with baffles for improved light utilization through the flashing light effect. *J. Biotechnol.* 92, 89–94. doi:10.1016/S0168-1656(01)00350-9
- del Rio-Chanona, E.A., Manirafasha, E., Zhang, D., Yue, Q., Jing, K., 2016. Dynamic modeling and optimization of cyanobacterial C-phycoerythrin production process by artificial neural network. *Algal Res.* 13, 7–15. doi:10.1016/j.algal.2015.11.004
- Ehrenfeld, J., Gertler, N., 1997. Industrial Ecology in Practice: The Evolution of Interdependence at Kalundborg. *J. Ind. Ecol.* 1, 67–79. doi:10.1162/jiec.1997.1.1.67
- Ehrenfeld, J.R., Chertow, M.R., 2003. Industrial symbiosis: the legacy of Kalundborg, in: Ayres, R.U., Ayres, L.W. (Eds.), *A Handbook of Industrial Ecology*. Cheltenham, UK, pp. 334–348.
- Enzing, C., Ploeg, M., Barbosa, M., Sijsma, L., 2014. Microalgae-based products for the food and feed sector: an outlook for Europe, JRC Scientific and Policy Reports. European Commission. doi:10.2791/3339
- European Federation of Biotechnology, 2016. European Federation of Biotechnology Section on Biochemical Engineering Science (ESBES) [WWW Document]. URL [http://www.efb-central.org/index.php/Main/section\\_on\\_biochemical\\_engineering\\_science](http://www.efb-central.org/index.php/Main/section_on_biochemical_engineering_science)
- European Medicines Agency, 2011. EMA-FDA pilot program for parallel assessment of Quality by Design applications.
- Faassen, S.M., Hitzmann, B., 2015. Fluorescence Spectroscopy and Chemometric Modeling for Bioprocess Monitoring 10271–10291. doi:10.3390/s150510271
- Fai, P.B., Grant, A., Reid, B., 2007. Chlorophyll a fluorescence as a biomarker for rapid toxicity assessment. *Environ. Toxicol. Chem.* 26, 1520.

doi:10.1897/06-394R1.1

- Farrelly, D.J., Everard, C.D., Fagan, C.C., McDonnell, K.P., 2013. Carbon sequestration and the role of biological carbon mitigation: A review. *Renew. Sustain. Energy Rev.* 21, 712–727. doi:10.1016/j.rser.2012.12.038
- Foley, P.M., Beach, E.S., Zimmerman, J.B., 2011. Algae as a source of renewable chemicals: opportunities and challenges. *Green Chem.* 13, 1399. doi:10.1039/c1gc00015b
- Galbács, G., 2015. A critical review of recent progress in analytical laser-induced breakdown spectroscopy. *Anal. Bioanal. Chem.* 407, 7537–7562. doi:10.1007/s00216-015-8855-3
- Gao, C., Xiong, W., Zhang, Y., Yuan, W., Wu, Q., 2008. Rapid quantitation of lipid in microalgae by time-domain nuclear magnetic resonance. *J. Microbiol. Methods* 75, 437–440. doi:10.1016/j.mimet.2008.07.019
- García-Camacho, F., López-Rosales, L., Sánchez-Mirón, A., Belarbi, E.H., Chisti, Y., Molina-Grima, E., 2016. Artificial neural network modeling for predicting the growth of the microalga *Karlodinium veneficum*. *Algal Res.* 14, 58–64. doi:10.1016/j.algal.2016.01.002
- Ge, Y., 2016. NIR Reflectance and MIR Attenuated Total Reflectance Spectroscopy for Characterizing Algal Biomass Composition. *Trans. Asabe* 59.
- Girard, J.-M., Deschênes, J.-S., Tremblay, R., Gagnon, J., 2013. FT-IR/ATR univariate and multivariate calibration models for in situ monitoring of sugars in complex microalgal culture media. *Bioresour. Technol.* 144, 664–668. doi:10.1016/j.biortech.2013.06.094
- Goueguel, C., McIntyre, D.L., Singh, J.P., Jain, J., Karamalidis, A.K., 2014. Laser-Induced Breakdown Spectroscopy (LIBS) of a High-Pressure CO<sub>2</sub>-Water Mixture: Application to Carbon Sequestration. *Appl. Spectrosc.* 68, 997–1003. doi:10.1366/13-07383
- Grobbelaar, J.U., 2013. Inorganic Algal Nutrition. *Handb. Microalgal Cult. Appl. Phycol. Biotechnol.* 123–133. doi:10.1002/9781118567166.ch8
- Grogard, F., Akhmetzhanov, A., Masci, P., Bernard, O., 2010. Optimization of a photobioreactor biomass production using natural light, in: 49th IEEE Conference on Decision and Control (CDC). IEEE, pp. 4691–4696. doi:10.1109/CDC.2010.5717185
- Guiry, M.D., 2012. HOW MANY SPECIES OF ALGAE ARE THERE? *J. Phycol.* 48, 1057–1063. doi:10.1111/j.1529-8817.2012.01222.x
- Hahn, D.W., Omenetto, N., 2012. Laser-induced breakdown spectroscopy (LIBS), part II: Review of instrumental and methodological approaches to

- material analysis and applications to different fields. *Appl. Spectrosc.* 66, 347–419. doi:10.1366/11-06574
- Haider, A.F.M.Y., Hedayet Ullah, M., Khan, Z.H., Kabir, F., Abedin, K.M., 2014. Detection of trace amount of arsenic in groundwater by laser-induced breakdown spectroscopy and adsorption. *Opt. Laser Technol.* 56, 299–303. doi:10.1016/j.optlastec.2013.09.002
- Havlik, I., Reardon, K.F., Ünal, M., Lindner, P., Prediger, A., Babitzky, A., Beutel, S., Scheper, T., 2013. Monitoring of microalgal cultivations with on-line, flow-through microscopy. *Algal Res.* 2, 253–257. doi:10.1016/j.algal.2013.04.001
- Havlik, I., Scheper, T., Reardon, K.F., 2016. Monitoring of microalgal processes. *Adv. Biochem. Eng. Biotechnol.* 153, 89–142. doi:10.1007/10\_2015\_328
- Havlik, I., Scheper, T., Reardon, K.F., 2015. Monitoring of Microalgal Processes, in: *Advances in Biochemical Engineering/biotechnology*. pp. 89–142. doi:10.1007/10\_2015\_328
- He, H.-J., Sun, D.-W., 2015. Microbial evaluation of raw and processed food products by Visible/Infrared, Raman and Fluorescence spectroscopy. *Trends Food Sci. Technol.* 46, 199–210. doi:10.1016/j.tifs.2015.10.004
- Hu, D., Liu, H., Yang, C., Hu, E., 2008. The design and optimization for light-algae bioreactor controller based on Artificial Neural Network-Model Predictive Control. *Acta Astronaut.* 63, 1067–1075. doi:10.1016/j.actaastro.2008.02.008
- Huang, H., Yu, H., Xu, H., Ying, Y., 2008. Near infrared spectroscopy for on/in-line monitoring of quality in foods and beverages: A review. *J. Food Eng.* 87, 303–313. doi:10.1016/j.jfoodeng.2007.12.022
- Huner, N., Öquist, P.A.G., Fathey, S., 1998. Energy Balance and Acclimation To Light and Cold 1385, 224–230.
- Huot, Y., Babin, M., 2010. Overview of Fluorescence Protocols: Theory, Basic Concepts, and Practice, in: *Chlorophyll a Fluorescence in Aquatic Sciences: Methods and Applications*. Springer Netherlands, Dordrecht, pp. 31–74. doi:10.1007/978-90-481-9268-7\_3
- ICH, 2009. Pharmaceutical Development Q8(R2): ICH Harmonised Tripartite Guideline, in: *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*. pp. 1–24.
- Jacobsen, N.B., 2008. Industrial Symbiosis in Kalundborg, Denmark: A Quantitative Assessment of Economic and Environmental Aspects. *J. Ind. Ecol.* 10, 239–255. doi:10.1162/108819806775545411

- Jung, S.-K., Lee, S.B., 2008. In Situ Monitoring of Cell Concentration in a Photobioreactor Using Image Analysis: Comparison of Uniform Light Distribution Model and Artificial Neural Networks. *Biotechnol. Prog.* 22, 1443–1450. doi:10.1021/bp0600886
- Justice, C., Brix, A., Freimark, D., Kraume, M., Pfromm, P., Eichenmueller, B., Czermak, P., 2011. Process control in cell culture technology using dielectric spectroscopy. *Biotechnol. Adv.* 29, 391–401. doi:10.1016/j.biotechadv.2011.03.002
- Khataee, A.R., Zarei, M., Pourhassan, M., 2010. Bioremediation of Malachite Green from Contaminated Water by Three Microalgae: Neural Network Modeling. *CLEAN - Soil, Air, Water* 38, NA-NA. doi:10.1002/clen.200900233
- Kliphuis, A.M.J., 2012. Metabolic modeling of *Chlamydomonas reinhardtii*: energy requirements for photoautotrophic growth and maintenance. *J. Appl. Phycol.* 24.
- Kok, B., 1956. Photosynthesis in flashing light. *Biochim. Biophys. Acta* 21, 245–258. doi:10.1016/0006-3002(56)90004-X
- Koller, M., Muhr, A., Braunegg, G., 2014. Microalgae as versatile cellular factories for valued products. *Algal Res.* 6, 52–63. doi:10.1016/j.algal.2014.09.002
- Komives, C., Parker, R.S., 2003. Bioreactor state estimation and control. *Curr. Opin. Biotechnol.* 14, 468–474. doi:10.1016/j.copbio.2003.09.001
- Ladner, D.A., Vardon, D.R., Clark, M.M., 2010. Effects of shear on microfiltration and ultrafiltration fouling by marine bloom-forming algae. *J. Memb. Sci.* 356, 33–43. doi:10.1016/j.memsci.2010.03.024
- Landgrebe, D., Haake, C., Höpfner, T., Beutel, S., Hitzmann, B., Scheper, T., Rhiel, M., Reardon, K.F., 2010. On-line infrared spectroscopy for bioprocess monitoring. *Appl. Microbiol. Biotechnol.* 88, 11–22. doi:10.1007/s00253-010-2743-8
- Latkoczy, C., Kägi, R., Fierz, M., Ritzmann, M., Günther, D., Boller, M., 2010. Development of a mobile fast-screening laser-induced breakdown detection (LIBD) system for field-based measurements of nanometre sized particles in aqueous solutions. *J. Environ. Monit.* 12, 1422. doi:10.1039/c002020f
- Lazic, V., Jovičević, S., 2014. Laser induced breakdown spectroscopy inside liquids: Processes and analytical aspects. *Spectrochim. Acta Part B At. Spectrosc.* 101, 288–311. doi:10.1016/j.sab.2014.09.006
- López-Rosales, L., Gallardo-Rodríguez, J.J., Sánchez-Mirón, A., Contreras-Gómez, A., García-Camacho, F., Molina-Grima, E., 2013. Modelling of



- multi-nutrient interactions in growth of the dinoflagellate microalga *Protoceratium reticulatum* using artificial neural networks. *Bioresour. Technol.* 146, 682–688. doi:10.1016/j.biortech.2013.07.141
- Lourenço, N.D., Lopes, J.A., Almeida, C.F., Sarraguça, M.C., Pinheiro, H.M., 2012. Bioreactor monitoring with spectroscopy and chemometrics: a review. *Anal. Bioanal. Chem.* 404, 1211–1237. doi:10.1007/s00216-012-6073-9
- Luttmann, R., Bracewell, D.G., Cornelissen, G., Gernaey, K. V., Glassey, J., Hass, V.C., Kaiser, C., Preusse, C., Striedner, G., Mandenius, C.-F., 2012. Soft sensors in bioprocessing: A status report and recommendations. *Biotechnol. J.* 7, 1040–1048. doi:10.1002/biot.201100506
- Maier, H.R., Dandy, G.C., 2001. Neural network based modelling of environmental variables: A systematic approach. *Math. Comput. Model.* 33, 669–682. doi:10.1016/S0895-7177(00)00271-5
- Malapascua, J., Jerez, C., Sergejevová, M., Figueroa, F., Masojídek, J., 2014. Photosynthesis monitoring to optimize growth of microalgal mass cultures: application of chlorophyll fluorescence techniques. *Aquat. Biol.* 22, 123–140. doi:10.3354/ab00597
- Mallick, N., 2002. Biotechnological potential of immobilized algae for wastewater N, P and metal removal: A review. *BioMetals* 15, 377–390. doi:10.1023/A:1020238520948
- Markou, G., Angelidaki, I., Georgakakis, D., 2012. Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. *Appl. Microbiol. Biotechnol.* 96, 631–645. doi:10.1007/s00253-012-4398-0
- Maxwell, D.P., Falk, S., Huner, N., 1995. Photosystem II Excitation Pressure and Development of Resistance to Photoinhibition (I. Light-Harvesting Complex II Abundance and Zeaxanthin Content in *Chlorella vulgaris*). *Plant Physiol.* 107, 687–694. doi:10.1104/pp.107.3.687
- Mayer, P., Cuhel, R., Nyholm, N., 1997. A Simple in vitro Fluorescence Method for Biomass Measurements in Algal Growth Inhibition Tests. *Water Resour.* 31, 2525–2531.
- Meng, Y., Yao, C., Xue, S., Yang, H., 2014. Application of Fourier transform infrared (FT-IR) spectroscopy in determination of microalgal compositions. *Bioresour. Technol.* 151, 347–354. doi:10.1016/j.biortech.2013.10.064
- Millie, D.F., Weckman, G.R., Young, W.A., Ivey, J.E., Carrick, H.J., Fahnenstiel, G.L., 2012. Modeling microalgal abundance with artificial neural networks: Demonstration of a heuristic “Grey-Box” to deconvolve

- and quantify environmental influences. *Environ. Model. Softw.* 38, 27–39. doi:10.1016/j.envsoft.2012.04.009
- Montingelli, M.E., Tedesco, S., Olabi, A.G., 2015. Biogas production from algal biomass: A review. *Renew. Sustain. Energy Rev.* 43, 961–972. doi:10.1016/j.rser.2014.11.052
- NAABB, 2014. National Alliance for Advanced Biofuels and Bioproducts.
- Nadadoor, V.R., De la Hoz Siegler, H., Shah, S.L., McCaffrey, W.C., Ben-Zvi, A., 2012. Online sensor for monitoring a microalgal bioreactor system using support vector regression. *Chemom. Intell. Lab. Syst.* 110, 38–48. doi:10.1016/j.chemolab.2011.09.007
- Neidhardt, J., Benemann, J.R., Zhang, L., Melis, A., 1998. Photosystem-II repair and chloroplast recovery from irradiance stress: relationship between chronic photoinhibition, light-harvesting chlorophyll antenna size and photosynthetic productivity in *Dunaliella salina* (green algae). *Photosynth. Res.* 56, 175–184. doi:10.1023/A:1006024827225
- Noack, K., Eskofier, B., Kiefer, J., Dilk, C., Bilow, G., Schirmer, M., Buchholz, R., Leipertz, A., 2013. Combined shifted-excitation Raman difference spectroscopy and support vector regression for monitoring the algal production of complex polysaccharides. *Analyst* 138, 5639. doi:10.1039/c3an01158e
- Nuzzo, G., Gallo, C., d’Ippolito, G., Cutignano, A., Sardo, A., Fontana, A., 2013. Composition and Quantitation of Microalgal Lipids by ERETIC 1H NMR Method. *Mar. Drugs* 11, 3742–3753. doi:10.3390/md11103742
- Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J., Cullen, M.R., Glass, A., Keogh, J.P., Meyskens, F.L., Valanis, B., Williams, J.H., Barnhart, S., Cherniack, M.G., Brodtkin, C.A., Hammar, S., 1996. Risk Factors for Lung Cancer and for Intervention Effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. *JNCI J. Natl. Cancer Inst.* 88, 1550–1559. doi:10.1093/jnci/88.21.1550
- Pittman, J.K., Dean, A.P., Osundeko, O., 2011. The potential of sustainable algal biofuel production using wastewater resources. *Bioresour. Technol.* 102, 17–25. doi:10.1016/j.biortech.2010.06.035
- Podder, M.S., Majumder, C.B., 2016. The use of artificial neural network for modelling of phycoremediation of toxic elements As(III) and As(V) from wastewater using *Botryococcus braunii*. *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* 155, 130–145. doi:10.1016/j.saa.2015.11.011
- Podevin, M., De Francisci, D., Holdt, S.L., Angelidaki, I., 2015. Effect of nitrogen source and acclimatization on specific growth rates of microalgae determined by a high-throughput in vivo microplate autofluorescence

- method. *J. Appl. Phycol.* 27, 1415–1423. doi:10.1007/s10811-014-0468-2
- Podevin, M., Fotidis, I.A., De Francisci, D., Møller, P., Angelidaki, I., 2017. Detailing the start-up and microalgal growth performance of a full-scale photobioreactor operated with bioindustrial wastewater. *Algal Res.* 25, 101–108. doi:10.1016/j.algal.2017.04.030
- Porep, J.U., Kammerer, D.R., Carle, R., 2015. On-line application of near infrared (NIR) spectroscopy in food production. *Trends Food Sci. Technol.* 46, 211–230. doi:10.1016/j.tifs.2015.10.002
- Pořízka, P., Prochazka, D., Pilát, Z., Krajcarová, L., Kaiser, J., Malina, R., Novotný, J., Zemánek, P., Ježek, J., Šerý, M., Bernatová, S., Krzyžánek, V., Dobranská, K., Novotný, K., Trtílek, M., Samek, O., 2012. Application of laser-induced breakdown spectroscopy to the analysis of algal biomass for industrial biotechnology. *Spectrochim. Acta - Part B At. Spectrosc.* 74–75, 169–176. doi:10.1016/j.sab.2012.06.014
- Pořízka, P., Prochazková, P., Prochazka, D., Sládková, L., Novotný, J., Petrílek, M., Brada, M., Samek, O., Pilát, Z., Zemánek, P., Adam, V., Kizek, R., Novotný, K., Kaiser, J., 2014. Algal biomass analysis by laser-based analytical techniques--a review. *Sensors (Basel)*. 14, 17725–17752. doi:10.3390/s140917725
- Posten, C., 2009. Design principles of photo-bioreactors for cultivation of microalgae. *Eng. Life Sci.* 9, 165–177. doi:10.1002/elsc.200900003
- Pragya, N., Pandey, K.K., Sahoo, P.K., 2013. A review on harvesting, oil extraction and biofuels production technologies from microalgae. *Renew. Sustain. Energy Rev.* 24, 159–171. doi:10.1016/j.rser.2013.03.034
- Rellán, S., Osswald, J., Saker, M., Gago-Martinez, A., Vasconcelos, V., 2009. First detection of anatoxin-a in human and animal dietary supplements containing cyanobacteria. *Food Chem. Toxicol.* 47, 2189–2195. doi:10.1016/j.fct.2009.06.004
- Schenk, J., Marison, I.W., von Stockar, U., 2008. pH prediction and control in bioprocesses using mid-infrared spectroscopy. *Biotechnol. Bioeng.* 100, 82–93. doi:10.1002/bit.21719
- Sharon Mano Pappu, J., Vijayakumar, G.K., Ramamurthy, V., 2013. Artificial neural network model for predicting production of *Spirulina platensis* in outdoor culture. *Bioresour. Technol.* 130, 224–230. doi:10.1016/j.biortech.2012.12.082
- Shastri, A. a, Morgan, J. a, 2005. Flux balance analysis of photoautotrophic metabolism. -Supplementary Material. *Biotechnol. Prog.* 21, 1617–26. doi:10.1021/bp050246d
- Shilton, A.N., Powell, N., Guieysse, B., 2012. Plant based phosphorus recovery

- from wastewater via algae and macrophytes. *Curr. Opin. Biotechnol.* 23, 884–889. doi:10.1016/j.copbio.2012.07.002
- Sieracki, M., Poulton, N., Crosbie, N., 2005. Automated isolation techniques for microalgae, in: Andersen, R.A. (Ed.), *Algal Culturing Techniques*. Burlington, MA.
- Skjelbred, B., Edvardsen, B., Andersen, T., 2012. A high-throughput method for measuring growth and loss rates in microalgal cultures. *J. Appl. Phycol.* 24, 1589–1599. doi:10.1007/s10811-012-9819-z
- Skogen Chauton, M., Røvik Størseth, T., Johnsen, G., 2003. High-resolution magic angle spinning <sup>1</sup> H NMR analysis of whole cells of *Thalassiosira pseudonana* (Bacillariophyceae): Broad range analysis of metabolic composition and nutritional value. *J. Appl. Phycol.* 15, 533–542. doi:10.1023/B:JAPH.00000004355.11837.1d
- Slegers, P.M., Wijffels, R.H., van Straten, G., van Boxtel, A.J.B., 2011. Design scenarios for flat panel photobioreactors. *Appl. Energy*. doi:10.1016/j.apenergy.2010.12.037
- Slocombe, S.P., Benemann, J.R., n.d. Microalgal production for biomass and high-value products.
- Smirnova, M., Mariia, 2016. CONCEPT OF A SUSTAINABLE FOOD-GROWING UNIT ON MARS.
- Sorokin, C., Krauss, R.W., 1962. Effects of Temperature & Illuminance on Chlorella Growth Uncoupled From Cell Division. *Plant Physiol.* 37, 37–42. doi:10.1104/pp.37.1.37
- Stöhr, C., Glogau, U., Mätschke, M., Tischner, R., 1995. Evidence for the involvement of plasma-membrane-bound nitrate reductase in signal transduction during blue-light stimulation of nitrate uptake in Chlorella saccharophila. *Planta* 197, 613–618. doi:10.1007/BF00191568
- Subhadra, B., Grinson-George, 2011. Algal biorefinery-based industry: an approach to address fuel and food insecurity for a carbon-smart world. *J. Sci. Food Agric.* 91, 2–13. doi:10.1002/jsfa.4207
- Subhadra, B.G., 2010. Sustainability of algal biofuel production using integrated renewable energy park (IREP) and algal biorefinery approach. *Energy Policy* 38, 5892–5901. doi:10.1016/j.enpol.2010.05.043
- Takahashi, M.B., Leme, J., Caricati, C.P., Tonso, A., Fernández Núñez, E.G., Rocha, J.C., 2015. Artificial neural network associated to UV/Vis spectroscopy for monitoring bioreactions in biopharmaceutical processes. *Bioprocess Biosyst. Eng.* 38, 1045–1054. doi:10.1007/s00449-014-1346-7

- Terry, K.L., 1982. Nitrate uptake and assimilation in *Thalassiosira weissflogii* and *Phaeodactylum tricornutum*: interactions with photosynthesis and with the uptake of other ions. *Mar. Biol.* 69, 21–30. doi:10.1007/BF00396956
- Tredici, M., Zittelli, G., 1998. Efficiency of sunlight utilization: tubular versus flat photobioreactors. *Biotechnol. Bioeng.* 57, 187–97.
- Trivedi, J., Aila, M., Bangwal, D.P., Kaul, S., Garg, M.O., 2015. Algae based biorefinery—How to make sense? *Renew. Sustain. Energy Rev.* 47, 295–307. doi:10.1016/j.rser.2015.03.052
- U.S. Department of Health and Human Services Food and Drug Administration, 2004. Guidance for Industry - PAT- A Framework for Innovative Pharmaceutical DEvelopment, Manufacturing, and Quality Assurance.
- Van Wageningen, J., De Francisci, D., Angelidaki, I., 2015a. Comparison of mixotrophic to cyclic autotrophic/heterotrophic growth strategies to optimize productivity of *Chlorella sorokiniana*. *J. Appl. Phycol.* 27, 1775–1782. doi:10.1007/s10811-014-0485-1
- Van Wageningen, J., Holdt, S.L., De Francisci, D., Valverde-Pérez, B., Plósz, B.G., Angelidaki, I., 2014. Microplate-based method for high-throughput screening of microalgae growth potential. *Bioresour. Technol.* 169, 566–572. doi:10.1016/j.biortech.2014.06.096
- Van Wageningen, J., Pape, M.L., Angelidaki, I., 2015b. Characterization of nutrient removal and microalgal biomass production on an industrial waste-stream by application of the deceleration-stat technique. *Water Res.* 75, 301–311. doi:10.1016/j.watres.2015.02.022
- Vanthoor-Koopmans, M., Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M., 2013. Biorefinery of microalgae for food and fuel. *Bioresour. Technol.* 135, 142–149. doi:10.1016/j.biortech.2012.10.135
- Vigani, M., Parisi, C., Rodríguez-Cerezo, E., Barbosa, M.J., Sijtsma, L., Ploeg, M., Enzing, C., 2015. Food and feed products from micro-algae: Market opportunities and challenges for the EU. *Trends Food Sci. Technol.* doi:10.1016/j.tifs.2014.12.004
- Vogt, F., White, L., 2015. Spectroscopic analyses of chemical adaptation processes within microalgal biomass in response to changing environments. *Anal. Chim. Acta* 867, 18–28. doi:10.1016/j.aca.2015.02.005
- Wagner, H., Liu, Z., Langner, U., Stehfest, K., Wilhelm, C., 2010. The use of FTIR spectroscopy to assess quantitative changes in the biochemical composition of microalgae. *J. Biophotonics* 3, 557–566.

doi:10.1002/jbio.201000019

- Wagner, I., Braun, M., Slenzka, K., Posten, C., 2015. Photobioreactors in Life Support Systems, in: Advances in Biochemical Engineering/biotechnology. pp. 143–184. doi:10.1007/10\_2015\_327
- Walsh, B.J., Rydzak, F., Palazzo, A., Kraxner, F., Herrero, M., Schenk, P.M., Ciais, P., Janssens, I.A., Peñuelas, J., Niederl-Schmidinger, A., Obersteiner, M., 2015. New feed sources key to ambitious climate targets. Carbon Balance Manag. 10, 26. doi:10.1186/s13021-015-0040-7
- Wicaksana, F., Fane, A.G., Pongpairaj, P., Field, R., 2012. Microfiltration of algae (*Chlorella sorokiniana*): Critical flux, fouling and transmission. J. Memb. Sci. 387–388, 83–92. doi:10.1016/j.memsci.2011.10.013
- Wijffels, R.H., Barbosa, M.J., 2010. An outlook on microalgal biofuels. Science 329, 796–9. doi:10.1126/science.1189003
- Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M., 2010. Microalgae for the production of bulk chemicals and biofuels. Biofuels, Bioprod. Biorefining 4, 287–295. doi:10.1002/bbb.215
- Wilde, E.W., Benemann, J.R., 1993. Bioremoval of heavy metals by the use of microalgae. Biotechnol. Adv. 11, 781–812. doi:10.1016/0734-9750(93)90003-6
- Yang, 2000. Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. Biochem. Eng. J. 6.
- Yang, Y., Park, Y., Cassada, D.A., Snow, D.D., Rogers, D.G., Lee, J., 2011. In vitro and in vivo safety assessment of edible blue-green algae, *Nostoc commune* var. *sphaeroides* Kützinger and *Spirulina plantensis*. Food Chem. Toxicol. 49, 1560–1564. doi:10.1016/j.fct.2011.03.052
- Yoo, S.J., Jeong, D.H., Kim, J.H., Lee, J.M., 2016. Optimization of microalgal photobioreactor system using model predictive control with experimental validation. Bioprocess Biosyst. Eng. 39, 1235–1246. doi:10.1007/s00449-016-1602-0
- Yoo, S.J., Jung, D.H., Kim, J.H., Lee, J.M., 2015. A comparative study of soft sensor design for lipid estimation of microalgal photobioreactor system with experimental validation. Bioresour. Technol. 179, 275–283. doi:10.1016/j.biortech.2014.12.029
- Yu, Y., Zhou, W., Qian, H., Su, X., Ren, K., 2014. Simultaneous Determination of Trace Lead and Chromium in Water Using Laser-Induced Breakdown Spectroscopy and Paper Substrate. Plasma Sci. Technol. 16, 683–687. doi:10.1088/1009-0630/16/7/09

Zittelli, G.C., Biondi, N., Rodolfi, L., Tredici, M.R., 2013. Photobioreactors for Mass Production of Microalgae. *Handb. Microalgal Cult. Appl. Phycol. Biotechnol.* 225–266. doi:10.1002/9781118567166.ch13

## 9 Papers

- I Podevin, M.**, De Francisci, D., Holdt, S.L., Angelidaki, I., 2015. Effect of nitrogen source and acclimatization on specific growth rates of microalgae determined by a high-throughput in vivo microplate autofluorescence method. *J. Appl. Phycol.* 27, 1415–1423. doi:10.1007/s10811-014-0468-2
- II Podevin, M.**, Fotidis, I.A., De Francisci, D., Møller, P., Angelidaki, I., 2017. Detailing the start-up and microalgal growth performance of a full-scale photobioreactor operated with bioindustrial wastewater. *Algal Res.* 25, 101–108. doi:10.1016/j.algal.2017.04.030
- III Podevin, M.**, Fotidis, I.A., Angelidaki, I., 2017. Microalgal process-monitoring based on high-selectivity spectroscopy tools: status and future perspectives. (Submitted to *Critical Reviews in Biotechnology*)

In this online version of the thesis, **paper I-III** are not included but can be obtained from electronic article databases e.g. via [www.orbit.dtu.dk](http://www.orbit.dtu.dk) or on request from

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